Production of Uniparental Embryonic Stem Cell Lines

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Abstract Embryonic stem cells, or induced pluripotent cells derived from somatic cells, can yield differentiated progeny with potential applicability for tissue repair. This chapter describes the generation of embryonic stem cells from gamete-derived uniparental embryos. These embryonic stem cells can be patient-derived and potentially histocompatible with the gamete donor. The production of uniparental embryos followed by derivation of embryonic stem cells can be accomplished without producing fertilized zygotes, an advantage that avoids some ethical issues. We describe methods for the generation of uniparental embryonic stem cells from mouse uniparental embryos. We also address evaluation of the integrity of the lines generated, an essential criterion in interpreting differentiation assays in vivo and in vitro.

Keywords Embryonic stem cells • Uniparental • Parthenogenetic • Androgenetic • Derivation • Pronuclear transfer

Introduction

Uniparental Embryonic Stem Cells: A Source of Pluripotent Stem Cells

Pluripotent embryonic stem (ES) cells have stimulated considerable interest for their potential use in therapeutic cell and tissue replacement ***(1–3)***. Aside from graft efficacy and safety concerns, one major hurdle for the potential application of differentiated ES cells in tissue repair is how to manage the requirement for immune

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compatibility ***(4–7)***. Potential approaches for the generation of patient-derived and autologous pluripotent stem cells include the derivation of ES cells from somatic cell nuclear transfer (SCNT) embryos, from uniparental embryos ***(8)***, and the induction of pluripotency in somatic cells (iPSCs) ***(9)***. This chapter describes the production of uniparental embryos and the derivation of embryonic stem cells thereof.

Mammalian uniparental embryos with only maternally (oocyte) or paternally (sperm) derived genomes fail early in development ***(10, 11)***; however, they typically reach at least the blastocyst stage and can give rise to ES cell lines ***(12, 13)***. Autologous uniparental ES cells derived using the gametes of a patient could be a potential source of tissue for cell replacement therapy. Unlike the extreme inefficiency observed with SCNT, murine uniparental embryos and ES cells can be obtained at rates similar to those of fertilized embryos ***(14, 15)*** (48% and 43% from one-cell stage, respectively), and human parthenogenetic (PG) ES cell lines have been derived ***(16–20)***. PG human ES cell lines are frequently major histocompatibility complex (MHC) matching to the oocyte donor ***(17)***. As uniparental conceptuses occur spontaneously but are not viable and can produce neoplasms (ovarian teratoma, hydatidiform moles) and even aggressive choriocarcinomas ***(21)***, using them to generate ES cells subjectively side steps some of the ethical perspectives associated with the destruction of potentially viable fertilized and SCNT embryos ***(22–24)***. iPSCs potentially address the concerns of destroying viable embryos. However, this approach requires considerable refinement to reduce the potential side effects of using genetic manipulation.

Origin and Generation of Uniparental Embryos and ES Cells

The most commonly known type of uniparental embryo is the PG embryo. Parthenogenesis is a type of gynogenesis, or generation of an organism with exclusively maternal genomes. It can be initiated by spontaneous or experimental activation of an unfertilized oocyte (Fig. 1a; top row). Diploidy of PG embryos can be achieved by suppressing extrusion of the second polar body during activation. Mouse experiments investigating the role of maternal and paternal genomes, including those requiring specific genotypes, often apply the transfer of maternal and paternal pronuclei between zygotes (pronuclear transfer, PNT; ***(25)***; Fig. 1b), producing gynogenetic (GG) embryos with two maternal genomes from different oocytes (Fig. 1b, top row), and androgenetic (AG) embryos with two paternal genomes (AG; Fig. 1b, bottom row). GG embryos develop similarly to PG embryos and are often used to study the consequences of two maternal genomes ***(26–28)***.

To avoid the ethical concerns of destroying a viable fertilized embryo, uniparental embryos by definition, and in practice, can be generated using only the genetic material of one individual of reproductive age of either sex, without fertilizing an

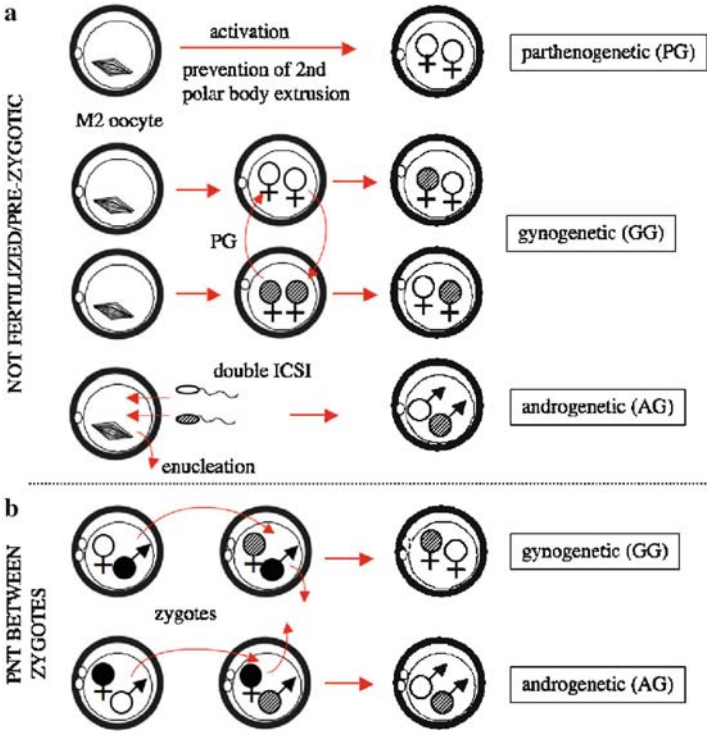

Fig. 1 Strategies for the production of uniparental embryos. (**a**) Approaches not involving fertilized embryos, by either activating a female's oocyte (parthenogenetic, PG), by exchange of pronuclei between PG embryos to produce gynogenetic (GG) embryos, or by injecting two sperm into an enucleated donor oocyte (androgenetic, AG). (**b**) Pronuclear transfer between zygotes ***(25)***, an approach often used in murine experiments, producing GG embryos or AG embryos with two maternal or paternal genomes from different zygotes, respectively.

intact oocyte (Fig. 1a). This includes PG embryos, as well as GG embryos that would result from the transfer of a pronucleus from one haploid or diploid PG embryo to another (if diploid PG are used, less oocytes are needed; Fig. 1a, middle row). For embryos derived only from paternal genomes, two sperm would be introduced into an enucleated donor oocyte by intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) at high sperm concentrations (AG; Fig. 1a, bottom row); this method has been established in the mouse and the bovine ***(29, 30)***. Based on the clinical success of ICSI, human AG embryos should be technically feasible. Although production of human uniparental embryonic stem cells would require the manipulation of human gametes, i.e., oocytes and sperm, it could be accomplished without the manipulation of zygotes (oocyte containing both sperm and egg genome).

Developmental Potential of Uniparental Embryos and Uniparental ES Cells

Androgenetic embryos rarely develop to or past early somite stages, with abundant trophoblast but retarded development of the embryo proper ***(10)***. Diploid parthenogenetic or gynogenetic embryos can occasionally develop to later somite stages but typically lack extraembryonic tissues ***(26, 31)***. This limited developmental potential of uniparental embryos ***(10, 11)*** is due to the lack of, or overexpression of, imprinted genes, i.e., genes that are preferentially expressed from only one parental allele ***(32)***. When combined with normal cells in composite animals (chimeras), uniparental embryonic cells and uniparental ES cells can contribute to various tissues, including the germline, although AG and PG derived cells exhibit bias in their differentiation into, and exclusion from, certain lineages ***(33–36)***. AG cells in developmental chimeras are frequently found in tissues derived from mesodermal lineages. Conversely, PG cell derivatives are often present in brain but rarely in mesodermal tissues such as skeletal muscle ***(12, 33, 34, 36–38)***. This contribution bias manifests later in development with marked differential in contribution to some tissues or elimination thereof at later fetal stages ***(33, 38)***. AG cells, even at low levels of contribution, cause severe defects and often lethality in chimeras ***(12, 33, 39)***, and readily transform in vitro ***(40)***. PG-derived cells contrast in that they exhibit a deficit in their capacity to proliferate at normal rates ***(40, 41)***. The basis for at least some of these developmental and proliferative defects arises from abnormal expression levels of imprinted genes that are involved in fetal growth regulation ***(14, 40, 42)***.

Therapeutic Potential of Uniparental ES Cell Derivatives

The therapeutic applicability of uniparental ES cells depends on their functional equivalence to normal ES cells, i.e., those that are derived from fertilized embryos. Consequences of genomic imprinting are potentially a major restriction to the therapeutic utility of uniparental ES cells. While PG ES cells have been proposed as a source of patient-derived therapeutic material ***(8)***, AG ES cells have not been similarly considered, in part because mouse experiments have shown that unlike PG cells, AG cells cause severe defects and frequent lethality when combined with normal cells in chimeras ***(12, 33, 39)***. AG ES cells, however, are pluripotent including germline transmission ***(12, 14, 33, 35, 43)***. Therapeutic utility of AG ES cell derivatives would include males of reproductive age into the patient pool, more than doubling the potential number of patients that could benefit from uniparental cell transplants. In contrast to developmental stages, the relevance of genomic imprinting for normal adult tissue function is largely unresolved ***(44)***. Our studies on hematopoietic transplantation of uniparental fetal liver cells and in vitro derivatives of uniparental ES cells suggest that genomic imprinting does not preclude a potential therapeutic applicability of uniparental cells in certain tissues ***(45)***.

We observed differentiation of AG and GG fetal liver derived cells into major blood lineages in adult recipients as defined by expression of CD4, B220, Gr-1, and Ter-119, and recipient mice were phenotypically normal when maintained in a specific pathogen-free (SPF) facility. We also observed engraftment and contribution of in vitro derivatives of uniparental ES cells in adults, using ectopic expression of the homeodomain protein HoxB4 in differentiating ES cells as an approach to promote formation of cells with a definitive hematopoietic phenotype ***(46)***. Contribution levels of uniparental ES cell derivatives in adult hosts were similar to those of normal ***(46)*** and nuclear-transfer–derived ***(47)*** ES cell derivatives, but due to the constitutive HoxB4 expression approach taken, we observed predominantly myeloid lineage differentiation ***(45)***.

Genomic Imprinting in Uniparental ES Cells

Genomic imprinting, defined as a bias in allele-specific expression of imprinted genes, is regulated in association with parent-specific imprinting marks that are set in the germ line, some of which involve differential methylation of regulatory regions. Uniparental ES cell lines are useful for studying genomic imprinting, however, with some limitations. Developmental potential, phenotypes of, and gene expression in uniparental ES cell chimeras, particularly those generated with AG ES cell lines ***(12, 48, 49)***, show that uniparental ES cells retain many aspects of genomic imprinting. The derivation and extended in vitro culture of ES cells can, however, change epigenetic marks and regulation of imprinted gene expression ***(50)***. ES cells, both those derived from normal and uniparental embryos, do not have an identified developmental state, are largely defined by their characteristics in in vitro culture, and can be epigenetically unstable with consequences on gene expression and thus differentiation potential ***(51, 52)***. For uniparental ES cell lines, AG lines appear to retain imprinting more faithfully than PG ES cell lines. For the latter, chimeras often exhibit phenotypic differences compared to PG aggregation chimeras, including the absence of growth deficits ***(49)*** and more widespread tissue contribution of PG ES cells ***(53)***. Consistent with our own observations that demonstrate less faithful conservation of gametic methylation marks in GG compared to AG ES cell lines ***(45)***, reactivation of paternally expressed genes in PG ES cell lines and changes in imprinted gene expression have been reported for PG ES cell lines and their derivatives in chimeras ***(54, 55)***.

Methods for the Derivation of Uniparental ES Cells

The methods for the derivation of uniparental ES cells include protocols for obtaining murine uniparental embryos, i.e., parthenogenetic activation for PG embryos, and pronuclear transfer for AG and GG embryos, culture to the blastocyst stage, and the

derivation of ES cell lines from blastocysts. This chapter emphasizes the description of protocol components relevant for uniparental ES cell production. For general techniques in embryo manipulation and ES cell culture, we refer to existing extensive protocols ***(56, 57)***.

Parthenogenetic Activation of Oocytes

To obtain diploid parthenogenetic embryos for the derivation of ES cell lines, oocytes are activated in the presence of cytochalasin B. The activation protocol is based on culture of oocytes in medium supplemented with strontium chloride in the absence of calcium chloride. This protocol works well for oocytes obtained from C57Bl6 × 129 or C57Bl6 × C3H F1 hybrid mice. Common alternative activation protocols include activation using ethanol exposure ***(57, 58)***.

Equipment and Reagents

- Dissection microscope; CO_2 incubator (5% CO_2, 37°C)
- Mouth or syringe controlled pipetting device for embryo handling
- Pasteur pipettes pulled to 0.3 mm outer diameter
- Dissection tools, fine forceps, 27-gauge syringes
- 35-mm suspension dishes (Corning 430588)
- 35-mm tissue culture dishes with four inner rings (Greiner Cellstar 627170)
- M2 medium (commercial supplier; or as described in ***(56)***)
- CZB culture medium (see below)
- Activation medium (see below): CZB culture medium without $CaCl_2$, supplemented with 10 mM $SrCl_2$ and cytochalasin B (5 μg/mL)
- Light mineral oil (Sigma); washed four times with millipore water (add twofold amount of sterile water to oil in sterile bottle/tissue culture flask, shake vigorously by hand several times, or on a shaker for 10–15 min, remove water; after last wash, add fresh water to oil in bottle).
- Hyaluronidase (Sigma H3884, 100 μg/mL in M2 medium; filter sterilize; store in 2–3 mL aliquots at −20°C)

Preparation of Culture and Activation Medium

Prepare media fresh from the following stock solutions on the day before activation:

Solution (concentration, volume)	Chemical	Grams
Solution 1 (10×; g in 500 mL)	KH_2PO_4	0.80
(add in the following order)	$MgSO_4 \times 7H_2O$	1.45
	NaCl	23.85
	KCl	1.80
	Na lactate	
	(60% syrup; Sigma L 4263)	29.24mL
	D-glucose	5.00
Solution 2 (10×; g to 48.5 mL)	$NaHCO_3$	1.055
	Add 1 mL of 5 mg/mL solution phenol red	
Solution 3 (100×; g to 50 mL)	Sodium pyruvate	0.145
Solution 4 (100×; g in 100 mL)	$CaCl_2 \times 2H_2O$	2.51
Solution 5 (200×)	Glutamine (200 mM; Gibco 25030-081)	
Solution 6 (100×)	Pen/Strep (100×; Gibco 15140-122)	

Storage: 1 and 4, filter sterilize, store at 4°C for 8 weeks; 2 and 3, make fresh; 5 and 6, store aliquots at −20°C.

Stock	Component	10 mL
	Water	7.65
1	Salts/sugars	1
2	HCO_3	1
3	Pyruvate	0.1
4	Ca	0.1
5	Glutamine	0.05
6	Pen/Strep	0.1

Preparation of CZB Culture Medium (10 mL):

Add 0.04 g BSA (Serologicals 81-003-2) per 10 mL, then filter sterilize. Equilibrate medium in tube or in culture drops overnight in CO_2 incubator. For culture drops, place 20 μL drops onto 35-mm suspension dish and cover with light mineral oil.

Preparation of CZB Medium for Activation (10 mL)

Combine water and stock solutions 1, 2, 3, 5, and 6 as described for CZB culture medium. Instead of stock solution 4, add 0.1 mL of a 1 M stock solution of $SrCl_2$ in water (10 mM final concentration of $SrCl_2$). Add BSA (0.04 g/10 mL), filter sterilize and equilibrate overnight in CO_2 incubator. On the day of activation, add 0.05 mL of 1 mg/mL cytochalasin B in dimethylsulfoxide (DMSO) (prepare sterile, store in aliquots at −80°C). Prepare culture drops as described above.

Parthenogenetic Activation of Unfertilized Oocytes

Induce ovulation in female mice (6–12 weeks old) by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG; 5 IU) followed 48 h later by intraperitoneal

injection of human chorionic gonadotropin (hCG; 5 IU). Sacrifice females 17 h post–hCG injection, dissect oviducts, place in large (0.5 mL drop) of hyaluronidase solution in the lid of a 35-mm culture dish and release oocyte–cumulus complexes from oviduct by opening the ampulla with a 27-guage needle while pinching it with fine forceps. Incubate for approximately 5–10 min at room temperature. Pick up oocytes using a mouth or syringe controlled fine Pasteur pipette and wash twice in a large drop of M2 medium (easiest in about 150 μL in a four-ring Greiner dish). Wash oocytes twice in 0.15 mL activation medium in a four-ring Greiner dish (dish preequilibrated in CO_2 incubator), then twice in 50 μL drops of activation medium covered with mineral oil. Place into preequilibrated culture drops made with activation medium and culture for 5–6 h in CO_2 incubator. Wash thoroughly in CZB culture medium (use large, preequilibrated culture drops, incubate several minutes in each drop) and discard fragmented oocytes, oocytes lacking a perivitelline space or of other unusual morphology or color. Place in preequilibrated CZB culture drops and culture for 3 days to the blastocyst stage. On the next day, score the number of embryos that have cleaved to the two-cell stage to assess activation. Remove fragmented embryos from the culture drops and discard.

Production of Androgenetic and Gynogenetic Embryos by Pronuclear Transfer

This approach involves the reciprocal exchange of male and female pronuclei between zygotes, resulting in embryos with two paternal (AG) or two maternal (GG) genomes from different zygotes. One pronucleus is removed with a micromanipulation pipette without disruption of the plasma membrane of the zygote, and the membrane-surrounded pronucleus (karyoplast) is subsequently fused with the recipient zygote, a method referred to as pronuclear transplantation ***(25)***. The experimental design for this approach needs to include genetic markers that allow it to distinguish AG or GG embryos after manipulation from the zygotes used for their production, such that errors (i.e., culture of nonmanipulated zygotes or false identification of pronuclei) become apparent. Such markers can include enhanced green fluorescent protein (eGFP; for example ubiquitously expressed transgene ***(59)***) and/or the intracellular biochemical marker glucose-phosphate-isomerase 1 (Gpi1). For example, when producing AG embryos with a desired genetic background of 129S1 (homozygous for the *a* allele of Gpi1), zygotes could be from the intercross of 129S1 male mice with C57Bl6 female mice (homozygous for the *b* allele of Gpi1). Only AG embryos and the resulting ES cell lines would be homozygous for the *a* allele of Gpi1, whereas those derived from fertilized embryos or manipulated embryos with a falsely introduced female pronucleus would be heterozygous for the *a* and *b* alleles, and embryos with two maternal genomes

would be homozygous for the *b* allele (see also characterization of newly derived ES cell lines in the section "Derivation of ES Cell Lines").

Equipment and Reagents

- Micromanipulation setup (see below)
- Glass capillaries (Clark Electromedical GC100-15 for holding, and Clark Electromedical GC100T-15 for enucleation pipettes)
- Pipette puller (P80 or better; Brown-Flaming)
- Capillary grinder (Bachhofer)
- Microforge (Defonbrune style)
- Micromanipulation chamber (i.e., 6 cm glass bottom Petri dish)
- Electrofusion setup: modified AC function generator or commercial electrofusion device
- Cell fusion chamber: two parallel electrodes with a distance that will sustain a field strength of 1.5 kV/mm
- HEPES-buffered medium for manipulation/washes (M2)
- Nocodazole (0.3 mg/mL in DMSO; 1,000×)
- Cytochalasin B (see the section "Preparation of Culture and Activation Medium")
- Fusion medium (see below)
- CZB culture medium (see the section "Preparation of Culture and Activation Medium")
- Silicon oil (200 fluid/20 centistokes)

The micromanipulation setup requires two three-dimension movement micromanipulators attached to an inverted microscope. To control meniscus movement in pipettes, 2-μm syringes are attached to holding and enucleation pipette instrument holders via thick-walled plastic tubing, and the system is filled with silicon oil.

Production of Media

Enucleation Medium

M2 supplemented with 0.3 μg/mL nocodazole and 5 μg/mL cytochalasin B. Make fresh on day of use.

Fusion Medium

0.3 M mannitol
0.1 mM $MgSO_4$
0.05 mM $CaCl_2$

Adjust pH at 7.4 and osmolarity to 280 mOsm. Filter, sterilize, and store at 4°C for up to 3 months.

Production of Pipettes

Pipettes are pulled from glass capillary tubing (150 mm long) on a pipette puller. Holding pipettes are pulled from thick walled tubing (Clark Electromedical GC100-15), are cut on a microforge to an O.D. of 150–180 μm and are polished with the microforge filament to an I.D. of 80–100 μm. Enucleation manipulation pipettes are made from thin walled tubing (Clark Electromedical GC100T-15), cut to an O.D. of 20–25 μm and ground on a capillary grinder to produce a 45° bevel. Pipettes are then washed in 25% hydrofluoric acid to sharpen the bevelled edge followed by spiking of the distal tip of the bevel on the microforge filament. All pipettes are bent on the microforge to facilitate positioning into the micromanipulation chamber.

Pronuclear Transfer

This procedure involves the following steps: identification of pronuclei in zygotes, removal of one pronucleus from a zygote, transfer of a karyoplast with a pronucleus from a different zygote under the zona pellucida, and subsequent electrofusion. For production of AG embryos, the female pronuclei are removed from zygotes and replaced with a second male pronucleus from a different zygote. For GG embryos, male pronuclei are replaced with female pronuclei. Depending on the desired genotype of AG or GG embryos, zygotes from the same or different intercrosses are used.

To recover zygotes, superovulate female mice as described above (see the section "Parthenogenetic Activation of Unfertilized Oocytes") and after hCG administration mate with male mice. On the next morning, check for the presence of copulatory plugs, and 15–17 h post-hCG, dissect oviducts from females with a copulatory plug and recover putative zygotes from the ampulla as described for oocytes (see the section "Parthenogenetic Activation of Unfertilized Oocytes").

Prior to manipulation, incubate presumptive zygotes for 20 min in enucleation medium. Visualize pronuclei and verify that both the male and female pronucleus are visible: The male pronucleus is initially smaller and peripheral in the oocyte cytoplasm, where the sperm entered the egg. Subsequently, the male pronucleus migrates toward the cortex and becomes larger. In contrast, the female pronucleus is typically proximal to the polar body. To simplify the manipulation phase, only zygotes with both pronuclei visible on a dissection microscope are selected for manipulation (Fig. 2a).

Manipulation is performed in groups of approximately 20 zygotes (estimating about 45–60 min for manipulation exposure). Position the zygote with the holding

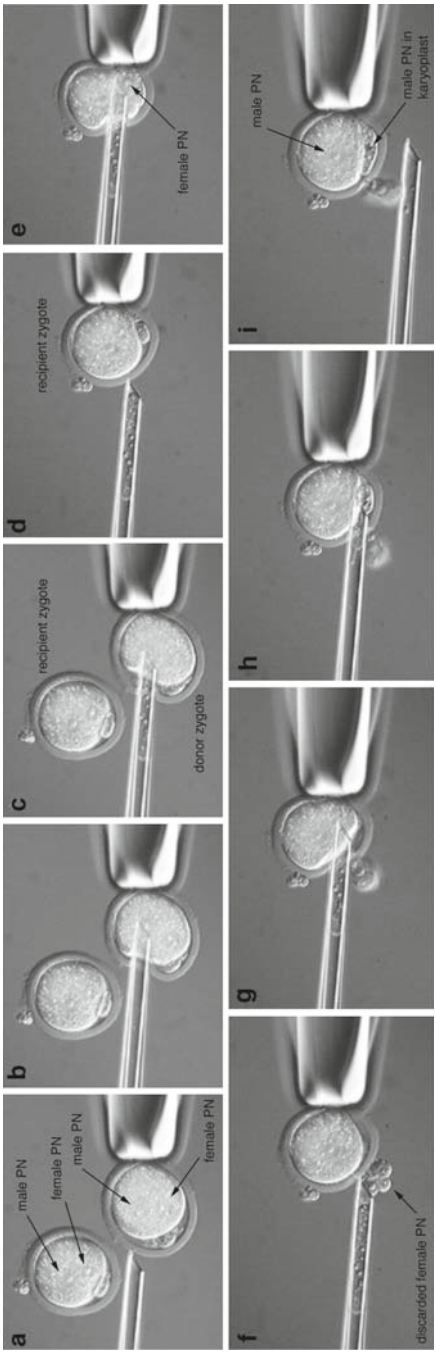

Fig. 2 Micromanipulation procedures to produce androgenetic (AG) embryos by pronuclear transfer. (**a**) Two zygotes at the pronuclear stage, arrows indicate male and female pronuclei (PN). (**b**) Positioning of the enucleation pipette for removal of the male PN from one zygote. (**c**) Removal of the karyoplast with the male pronucleus from the donor zygote by suctioning into the pipette. (**d**) Positioning of the recipient zygote for removal of the female PN. **(e)** Removal of the female PN from the recipient zygote. **(f)** The female pronucleus is expelled from the pipette. **(g, h)** Insertion and placement of the karyoplast with the male pronucleus from the donor zygote subzonally of the recipient zygote from which the female pronucleus has been removed. **(i)** AG construct with two male PN, one subzonally, ready for electrofusion.

pipette, and using a 20–25 μM O.D. pipette beveled to 45 degrees, remove male for production of AG (Fig. 2b, c), or female pronucleus for production of GG embryos. With this pronucleus in the pipette, the opposite parental pronucleus is removed from another zygote (Fig. 2d, e), expelled from the pipette (Fig. 2e), and the transplant pronucleus inserted subzonally immediately afterward (Fig. 2g, h). The successfully manipulated zygotes can be readily identified within the batch at the end of each group manipulation based on the presence of the karyoplast subzonally (Fig. 2i).

To fuse subzonal karyoplasts with the manipulated zygotes, the constructs are first equilibrated in electrofusion medium for 2 min. Constructs are then placed in an AC field to polarize and align karyoplasts and to achieve close contact (AC stimulus at 500–1,000 kHz, sine wave, 0–20 V pp). Once alignment and juxtaposition are achieved, a DC pulse is generated (1–1.5 kV/cm, 1–2 pulses, 50–100 ms interval, 20–100 ms duration). Optimal fusion parameters vary between chambers and electrodes and can be determined by fusing two-cell stage embryos and assessing fusion rate versus blastocyst development ***(60)***.

After fusion treatment, constructs are immediately washed in CZB culture medium (as described above for oocytes post activation in the section "Parthenogenetic Activation of Unfertilized Oocytes") and cultured in CZB culture drops to the blastocyst stage.

Derivation of ES Cell Lines

Equipment and Reagents

- 96, 48, 24, 12, 6-well tissue culture dishes (Falcon)
- Pulled Pasteur pipettes, lightly flame polished for embryo handling
- Feeder cells for ES cell derivation: STO fibroblasts; available from ATCC (CRL-1503). STO cells that have been stably transfected with a neo^r vector and an LIF expression vector (SNL cells) are courtesy of Allan Bradley and Elizabeth Robertson
- M2 medium (see above)
- Tyrode's solution, acidic
- Media and solutions described in the section "Preparation of Solutions and Media"

Preparation of Solutions and Media

ES Cell Medium

- 500 mL DMEM (Specialty Media/Chemicon EmbryoMax SLM-220-B; without l-glutamine and Na-pyruvate; with 4,500 mg/L glucose, 2,250 mg/L Na Bicarb)
- 6 mL nonessential amino acids (100×; Gibco 11140-050)

- 6 mL Pen/Strep (100×; Gibco 15140-122)
- 6 mL L-glutamine (100×; Gibco 25030-081)
- 0.6 mL β-mercaptoethanol (1,000×; Gibco 21958-023)
- 75 mL fetal bovine serum (Hyclone defined FBS; SH30070.03)

Store at 4°C. After 3 weeks, replenish glutamine and 2-mercaptoethanol from the respective stock solution according to the amount of medium left in bottle. If using mouse primary embryonic fibroblasts (MEF) or STO cells as feeder layers, add LIF (Chemicon Esgro® LIF ESG1106 or 1107) to the medium (500 U/mL final).

Medium for Feeder Cells

Same composition as ES cell growth medium, but lower concentration of FBS (35 mL FBS per 500 mL, i.e., 7%), also use DMEM from Gibco (11965). Store at 4°C.

1× Dulbecco's Phosphate Buffered Saline (DPBS)

Dilute from 10× stock (Gibco 14200-075; without calcium or magnesium).

Mitomycin C (MMC)

Dissolve 2 mg of mitomycin C (Sigma M 0503) in 5.0 mL of 1× DPBS (40× stock). Store 200-µL aliquots in sterile tubes at −80°C; add one aliquot to 10 mL medium for treatment (8 µg/mL final).

PBS/Gelatin

Add 1 g gelatin (Sigma G 2500) to 1,000 mL 1× DPBS in glass bottle, then autoclave. Store at 4°C after opening; can keep for 2 months.

Trypsin/EDTA

0.25% Trypsin (Sigma T 4799), 1 mM EDTA, 1× DPBS.

To prepare 1,000 mL	Trypsin	2.5 g
	EDTA 500 mM stock	2 mL
	10 × DPBS stock	100 mL

Add water ad 200 mL, filter sterilize, aliquot 10 mL of the resulting 5× stock into 50 mL tubes and store at −20°C. To produce 50 mL working solution, add 39.5 mL water and 0.5 mL of filter sterilized 5% (w/v) BSA (Sigma A 9647 in water) to thawed stock. Store the working solution at 4°C for 1–2 weeks.

Cell Freezing Solutions

- Solution I: 50% (v/v) FBS in DPBS+ (1×; Gibco 14287-080).
- Solution II: mixture of 20 mL 1× DPBS and 5 mL DMSO (Sigma D 2650).
- Store at 4°C. Discard after 14 days.

Derivation of ES Cell Lines

The method described has been adapted from various published protocols, including ***(58, 61)***. An overview of the procedure is outlined in Fig. 3. Blastocyst stage embryos from which the zona pellucida has been removed, are placed in individual wells of a 96-well plate and are cultured for 3 days to form outgrowths. To disaggregate these outgrowths (passage 0), the whole well is treated with trypsin, and all cells from each well of the 96-well plate are transferred to a well of a 48-well plate. An alternative method to this is picking and disaggregating only the inner cell mass of the outgrowth ***(58, 61)***. Culture expansion is continued by treating entire wells with trypsin and transferring them into gradually larger dishes. Again, an alternative method at passage 1 is picking individual ES cell colonies rather than transferring the whole well. All ES cell derivation and culture is performed on wells covered with feeder layers.

Preparation of Feeder Layers

Grow STO or SNL on 15 cm dish to confluency. Aspirate medium and replace with feeder medium containing MMC (1×) and incubate for 2 h at 37°C in CO_2 incubator. Wash plate several times with PBS, trypsinize cells, and determine cell count. Plate cells on gelatin-treated culture dishes (resuspend cells at a concentration of 1×10^7 cells/mL, plate 3.5 μL per well of a 96-well plate, 8.75, 17.5, 35, and 70 μL per well of a 48-, 24-, 12-, and 6-well plate, respectively). For gelatin-treatment of dishes, cover bottom of wells with gelatin/PBS and incubate at 4°C for 1 h or at room temperature for 20 min. Aspirate gelatin completely (tilt plates when aspirating) and add feeder medium to the well.

Zona Removal

Place blastocyst stage embryos into drop of M2 medium. Transfer into large drop (200 μL) of Tyrode's solution and observe dissolving of the zona pellucida through a dissection microscope. Remove embryos from Tyrode's as soon as zona is dissolved, wash several times in M2 medium.

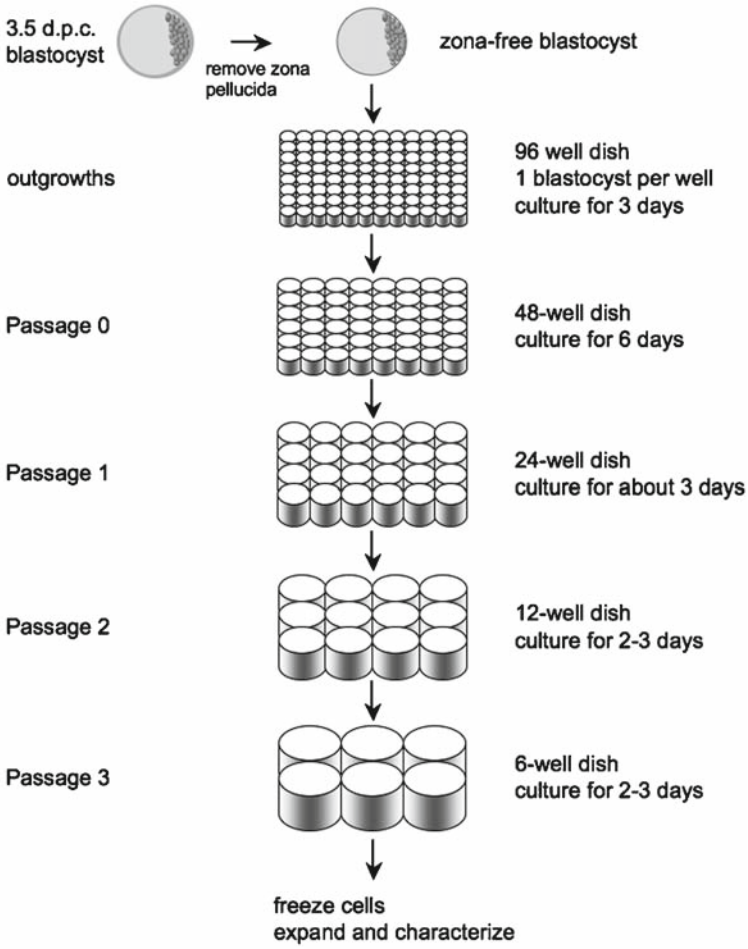

Fig. 3 Overview of embryonic stem (ES) cell derivation. Passaging between stages is performed by trypsin treatment, and the whole contents of each well transferred to a fresh well with feeder cells. After passage 1, subsequent passaging may have to be adjusted to the density of the ES cell colonies. Wells with only few colonies should be passaged onto the same size well (24 well) to increase colony density.

Outgrowths and Early Passage to Establish ES Cell Lines

Place zona-free blastocyst stage embryos individually onto wells of a 96-well plate covered with STO or SNL feeder cells. Culture in ES cell medium for 3 days, until the inner cell mass has formed a large, often mushroom-shaped outgrowth. Trypsinize the whole well (passage zero, p0): aspirate medium, rinse with 1× DPBS, rinse briefly with trypsin, and then add small amount of trypsin such that

the bottom of the well is just covered. Place into incubator for approximately 3–5 min. Add ES cell medium and, using Gilson pipette, repeatedly pipette up and down to break up clumps and obtain a cell suspension. Transfer cells from each well onto a fresh well of a 48-well plate with feeder cells. Culture cells in ES cell medium for 6–7 days. Colonies appearing to be ES cell-like can form earlier but should be ignored until the 6–7 days after passage. Trypsinize each well as described (passage 1, p1), and plate onto wells of a 24-well plate. Distinct ES cell colonies will appear in about 2–3 days. Continue passaging, using a 1:2 to 1:3 split to obtain sufficient density at early passages (see Fig. 3). Typically, new lines will be in a well of a 6-well plate at passage 3 or 4 and can be frozen (two vials per well of a 6-well plate) or expanded for further characterization. If the density of colonies is low, for example at passage 1 or 2, passage cells once on the same size well, i.e., perform passage 2 onto a 24 well or passage 3 onto a 12 well. Once lines are established, ES cells are split 1:3 to 1:6 every 2 days, when they are at approximately 70% confluency.

Freezing and Thawing ES Cells

Freezing. ES cells should be at about 70% confluency; change medium 2–3 h before freezing. Dissociate cells by trypsinization, add 2 mL ES cell maintenance medium and collect by brief centrifugation (1,100 rpm, 3 min). Resuspend cell pellet in half of the desired final volume of freezing solution I, then slowly, drop by drop, add an equal volume of freezing solution II while mixing carefully by gently flicking tube. Transfer into cryovial and place into –80°C freezer in a cooling device providing a controlled freezing rate (StrataCooler® or similar); then transfer to liquid nitrogen storage after 24 h. Freeze two vial with 500 μL volume each from one well of a 6-well plate or four vials from one 6-cm dish.

Thawing. Place vial in 37°C water bath to thaw, transfer contents into 15-mL tube. Slowly add ES cell medium to about 3–4 mL, collect cells by brief centrifugation, resuspend in ES cell maintenance medium, and plate onto wells with feeder cells.

Characterizing Newly Derived ES Cell Lines

The characterization of newly derived ES cell should include analysis of chromosome number for all lines, verification of uniparental origin for AG and GG ES cell lines derived from embryos generated by pronuclear transfer between zygotes, and analysis of Y-chromosome status for AG ES cell lines. Other analyses can include but are not limited to verification of genomic integrity (i.e., chromosome duplication or translocation) by G-banding of metaphase spreads; single nucleotide polymorphisms (SNP) analysis to determine the degree of homozygosity, which also serves as verification of uniparental origin, for example, in PG lines ***(62)***. The ability of ES cell lines to contribute in chimeras can only be ascertained by performing

blastocyst injection (for protocols, see ***(56, 63, 64)***), and subsequent analysis of the presence of ES cell derived cells in fetal stage or postnatal chimeras. Markers to identify ES cells in chimeras include eGFP (ES cells derived from eGFP transgenic mouse strains), or the presence of mouse strain-specific glucose-phosphate-isomerase 1 isoforms that can be ascertained by electrophoretic analysis of tissue samples (Gpi-1 isozyme analysis) ***(65)***.

Determine chromosome number by making metaphase spreads (for protocol see ***(56)***). To verify uniparental origin using Gpi-1 isoforms, collect about five large ES cell colonies with a pulled Pasteur pipette, freeze-thaw twice in 20 μL water and perform Gpi-1 electrophoresis. (If feeders are included in the sample, STO feeder cells are homozygous for the B isoform.) To determine Y-chromosome status, perform polymerase chain reaction (PCR) on genomic DNA: isolate genomic DNA from ES cells, or collect about ten large colonies as described above and digest at 55°C for 1–2 h in a shaker block in 10× volume of digest buffer (100 mM Tris–HCl pH 8.0, 0.5% (v/v) Tween 80, 0.5% (v/v) NP-40 with 100 mg/mL proteinase K). Dilute viscous digest 1:40 and use 2 μL in a 20 μL PCR reaction. Primers for the murine Zfy gene are 5′-CTC ATG CTG GGA CTT TGT GT-3′ and TGT GTT CTG CTT TCT TGG TG-3′, amplifying a fragment of 406 base pairs length. Verify presence and accessibility of genomic DNA in each sample by also performing PCR for a housekeeping gene such as β-actin.

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