Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of Embryonic Stem-Cell Lines from Human Blastocysts. N Engl J Med 2004;350:1353-6.

Supplementary Appendix 1

This appendix has been provided by the authors to give readers addditional information about their work.

MATERIALS AND METHODS:

DERIVATION AND CULTURE OF HES CELLS.

Supernumerary frozen human cleavage stage or blastocyst embryos produced by in vitro fertilization for clinical purposes were donated after informed consent and institutional review board approval. Embryos were cultured to the blastocyst stage and the zona pellucida removed by digestion with acid tyrodes (SPECIALTY MEDIA CAT#MR004-D), followed by immunosurgery using rabbit anti-human RBC antibodies (INTER CELL TECHNOLOGIES CAT#0183) and guinea pig sera complement (SIGMA, CAT#S-1639). The isolated ICMs were cultured on a feeder layer of mitomycin C (SIGMA CAT#M-0503) mitotically inactivated mouse embryonic fibroblasts (from day 12.5 post coitum fetuses of the ICR strain) plated on gelatin (SIGMA CAT#G1890) coated tissue culture plates at a density of approximately 50,000 cells/cm^2. The culture medium consisted of 80% Knockout DMEM (INVITROGEN GIBCO CAT#10829), 8% or 10% KO-Serum Replacement (INVITROGEN GIBCO CAT#10828-018), 8% or 10% Plasmanate (BAYER, CAT# 0026-0613-20), 5% fetal calf serum (HYCLONE CAT#SH30070.03), 2mM Glutamax-I (INVITROGEN GIBCO CAT#35050-061), 1% non-essential amino acids (INVITROGEN GIBCO CAT # 11140050), 50 units/mL penicillin and 50 ug/mL streptomycin (INVITROGEN GIBCO CAT#15070-063), 0.055mM beta-mercaptoethanol (GIBCO CAT# 21985-023), 12ng/mL recombinant hLIF (CHEMICON INTERNATIONAL CAT# LIF1010), and 5ng/mL bFGF (INVITROGEN CAT# 13256-029). 6 to 10 days after the initial plating, ICM-like clumps were mechanically dissociated and replated on fresh feeder layers. After the first passage, FCS was omitted from the culture media. The resulting colonies were further passaged by mechanical dissociation until there were sufficient cells (usually 1x10^5 cells) to passage enzymatically with 0.05% trypsin/EDTA (INVITROGEN GIBCO CAT# 25300-054). Thereafter hES cells were propagated by enzymatic dissociation with 0.05% trypsin/EDTA. All 17 hES cell lines were cryopreserved in freezing media consisting of 90% FCS and 10% DMSO, although cryopreservation in hES culture media plus 10% DMSO resulted in similar recovery upon thawing.

CHARACTERIZATION OF UNDIFFERENTIATED MOLECULAR MARKERS.

Subconfluent cells were characterized enzymatically and immunohistochemically using antibodies against markers of undifferentiated hES cells. Alkaline phosphatase staining of cells was performed according to the manufacturer's specifications. Colonies were fixed in 4% paraformaldehyde (PFA) for 15 minutes at 4 degrees Celsius, washed twice with PBS pH 7.5, and then blocked in PBS pH 7.5 supplemented with 5% normal donkey serum and 0.01% triton-X 100 for 1 hour at RT. Primary antibodies were added and incubated overnight at 4 degrees Celsius (mouse monoclonal Oct-4 C-10 Santa Cruz CAT# SC-5279, rat monoclonal SSEA-3 Developmental Hybridoma Studies Bank CAT# MC-631, mouse monoclonal SSEA-4 Developmental Hybridoma Studies Bank CAT# MC-813, mouse monoclonal TRA-1-60 Chemicon International CAT# MAB4360, or mouse monoclonal TRA-1-81 Chemicon International CAT# MAB4381). The cells were washed three times in PBS pH 7.5 for 10 minutes at RT and then fluorescent secondary antibodies (Rhodamine-Red-X donkey anti-mouse IgG Jackson ImmunoResearch Cat# 715-295-150 or Rhodamine-Red-X goat anti-rat Jackson ImmunoResearch CAT# 112-295-102) were added and incubated for 45 minutes at RT. The cells were washed three times in PBS pH 7.5 (the first two washes contained DAPI at a final concentration of 1µg/mL) and either imaged by indirect immunofluorescence or mounted (Aquapolymount, Polysciences Inc. CAT# 18606) and analyzed by confocal microscopy (Zeiss LCM 510).

KARYOTYPE ANALYSIS.

hES cells were grown to subconfluency at which point demecolcine (Sigma #D1925) was added to a final concentration of 0.1ug/mL for 1 hour. Cells were then trypsinized, pelleted by centrifugation at 1000xG for 5 minutes, resuspended in 5 mLs of 0.075 KCl, and incubated for 10 minutes at RT. After pelleting of cells again by centrifugation, the supernatant was removed and ice cold fresh fixative comprised of 1 part acetic acid to three parts methanol was added drop-wise to a final volume of 10mLs. Cells were fixed for 10 minutes at -20 degrees Celsius, pelleted by centrifugation, supernatant removed and fresh fixative added to a concentration of approximately 10^7 cells/mL. Metaphases from cells prepared in this manner were analyzed by GTG-banding. Approximately 20 metaphase spreads were counted and 5 metaphases analyzed for each hES cell line.

DETERMINATION OF POPULATION DOUBLING TIME.

20 wells of a 24 well tissue culture plate with mitotically inactivated MEF feeders were seeded with approximately 20,000 hES cells/well. 24 hours later, 4 wells of MEF feeders and 4 wells of hES cells plus feeders were trypsinized to a single cell suspension and cells from each well counted twice with a hemacytometer. The eight counts for the MEF feeders were averaged and subtracted from the average of the eight counts that corresponded to those wells with hES cells. This value was used as a baseline (t=0) for the doubling time assay. This procedure was repeated with 4 wells of hES cells at 48 hours, 72 hours, and 96 hours after seeding (low passage hES cells are analyzed at 72 hours, 120 hours, and 168 hours after seeding). In addition, one well of hES cells was alkaline phosphatase (Vector Labs Cat#SK-5100) stained at each time point and colony morphology and uniformity of staining assessed. Data were subsequently plotted and analyzed using Microsoft Excel.

IN VITRO DIFFERENTIATION (EB FORMATION).

In order to reduce the amount of MEFs in the hES cell cultures, cells were first split to gelatin coated tissue culture dishes (usually at ratios of 1:3 to 1:6) and grown for 6-10 days until 80-90% subconfluent. To induce formation of EBs, hES cells growing on gelatin coated plates were treated with trypsin at RT for 2-3 minutes and replated (1:1 ratio) onto ultra low cluster 6 well flat bottom dishes (Costar #3471) to allow aggregation and prevent adherence to the plate. Human EBs were grown in the same culture media as undifferentiated hES cells except that it lacked Plasmanate, hLIF, bFGF and had 20% KO-Serum Replacement. The EBs were cultured for 30 days and then fixed in 4% PFA, incubated in 30% sucrose, embedded in OCT and cryosectioned (10-15uM sections).

TERATOMA FORMATION.

Cells were dissociated with trypsin and then injected subcutaneously with a 23G needle into SCID or Nude-Swiss Webster mice (~$2x10^6$ cells per site). After teratomas had grown to a size greater than 3mM in diameter (usually 10 to 12 weeks), mice were euthanized and lesions surgically removed. Teratomas were fixed in 4% PFA overnight at 4 degrees Celsius, incubated overnight in 30% sucrose, embedded in OCT and cryosectioned (10-15uM sections).

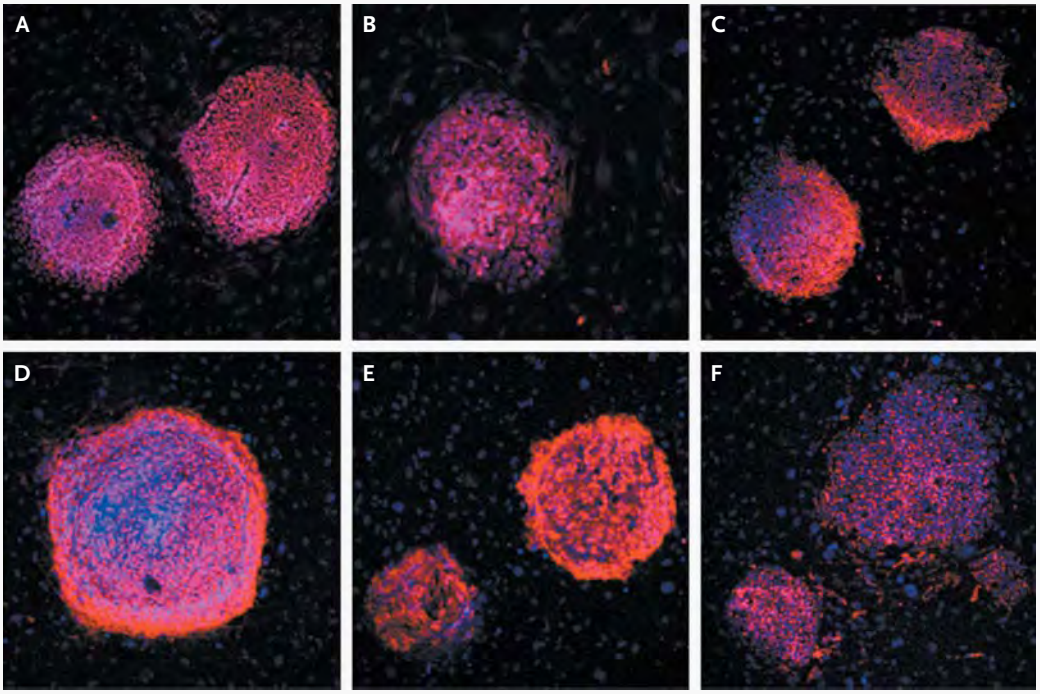
DIFFERENTIATED MARKER STAINING.

Cryosections were air dried and then washed three times for ten minutes each in PBS pH7.5 to remove excess OCT. The sections were then blocked in PBS pH 7.5 supplemented with 5% normal donkey serum and 0.01% triton-X 100 for 1 hour at RT. Primary antibodies were added and incubated overnight at 4 degrees Celsius (rabbit anti-human Alpha-1-Fetoprotein DAKO CAT# A0008, mouse monoclonal anti-rat Class III β-tubulin (TUJ1) Covance CAT# MMS-435P, or mouse monoclonal anti-chicken Myosin Heavy Chain Developmental Hybridoma Studies Bank CAT# MF 20). Sections were then washed three times in PBS pH 7.5 for 10 minutes at RT and then fluorescent secondary antibodies (Rhodamine-Red-X donkey anti-mouse IgG Jackson ImmunoResearch Cat# 715-295-150 or Rhodamine-Red-X donkey anti-rabbit Jackson ImmunoResearch Cat# 711-295-152) were added and incubated for 45 minutes at RT. Sections were washed three times in PBS pH 7.5 (the first two washes contained DAPI at a final concentration of 1µg/mL), mounted (Aquapolymount, Polysciences Inc. CAT# 18606), coversliped, and analyzed by confocal microscopy (Zeiss LSM 510).

Supplementary Table 1.

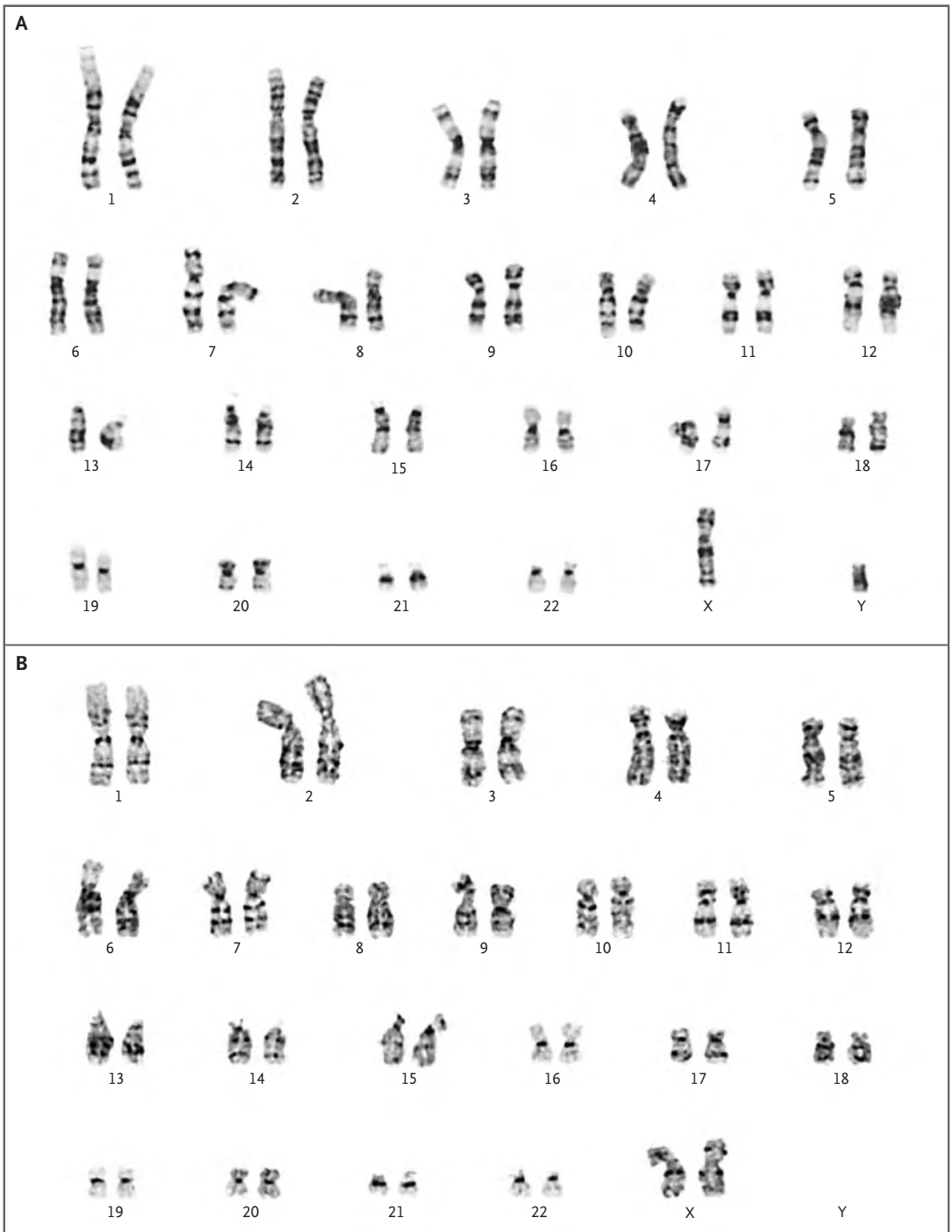
HUES cell line #	Blastocyst Score	Undifferentiated Staining (positive for Oct 4, SSEA 3/4, TRA-1-60/81 and AP)	Embryoid Bodies (stained positive for 3 germ layers)	Teratoma (stained positive for 3 germ layers)	Karyotype	Doubling Time	Frozen and Thawed	Expanded	Passage Achieved (# of pop. dblngs)
1	4AB	√	√	√	p20-46,XX p30-46,XX p39-46,XX,2q+	p43; 24hrs	√	40 vial p27 60 vials p16 30 vials p15	p56 (~140)
2	3BB	√	√	√	p48-46,XX p50-46,XX	p38; 24hrs	√	50 vials p16	p59 (~147)
3	4AC	√	√	√	p23-46,XY p33-47,XY,+12/46,XY p34-47,XY,+12/46,XY	p34; 60hrs	√	50 vials p26	p52 (~130)
4	3BB	√	√	√	p20-46,XY p22-46,XY p29-47,XY,+12/46,XY	p35; 48hrs	√	65 vials p21	p51 (~127)
5	3BC	√	√	√	p14-*46,XX, inv9	p15; 72hrs	√	80 vials p12	p28 (~84)
6	4AC	√	√	√	p12-46,XX	p19; 24hrs	√	80 vials p17	p54 (~132)
7	4BB	√	√	√	p10-46,XY	p13; 48hrs	√	75 vials p11	p26 (~78)
8	3AA	√	√	√	p10-46,XY	p19; 48hrs	√	50 vials p21	p23 (~69)
9	3AA	√	√	√	p9-*46,XX,inv9	p11; 24hrs	√	50 vials p13	p22 (~66)
10	3CA	√	√	√	p7-46,XY	p19; 72hrs	√	75 vials p10	p21 (~63)
11	4CB	√	√	√	p10-46,XY	N.D.	√	50 vials p13	p18 (~54)
12	4AC	√	√	√	p12-46,XX p15-46,XX	N.D.	√	75 vials p13	p24 (~72)
13	3AA	√	√	√	p11-46,XY	p11; 24hrs	√	80 vials p15	p18 (~54)
14	4AA	√	√	√	p10-46,XX p11-46,XX	p14; 48hrs	√	40 vials p16	p18 (~52)
15	3CC	√	√	√	p7-46,XX	p10; 48hrs	√	30 vials p12 60 vials p13	p17 (~51)
16	4AA	√	√	N.D.	p8-46,XY p9-46,XY	p9; 48 hrs	√	56 vials p14 80 vials p15	p18 (~54)
17	4AA	√	N.D.	√	p13-46,XY	N.D.	√	40 vials p15 60 vials p16	p17 (~51)

* inv(9)(p11q13) is a frequently observed chromosomal variant of no clinical significance.



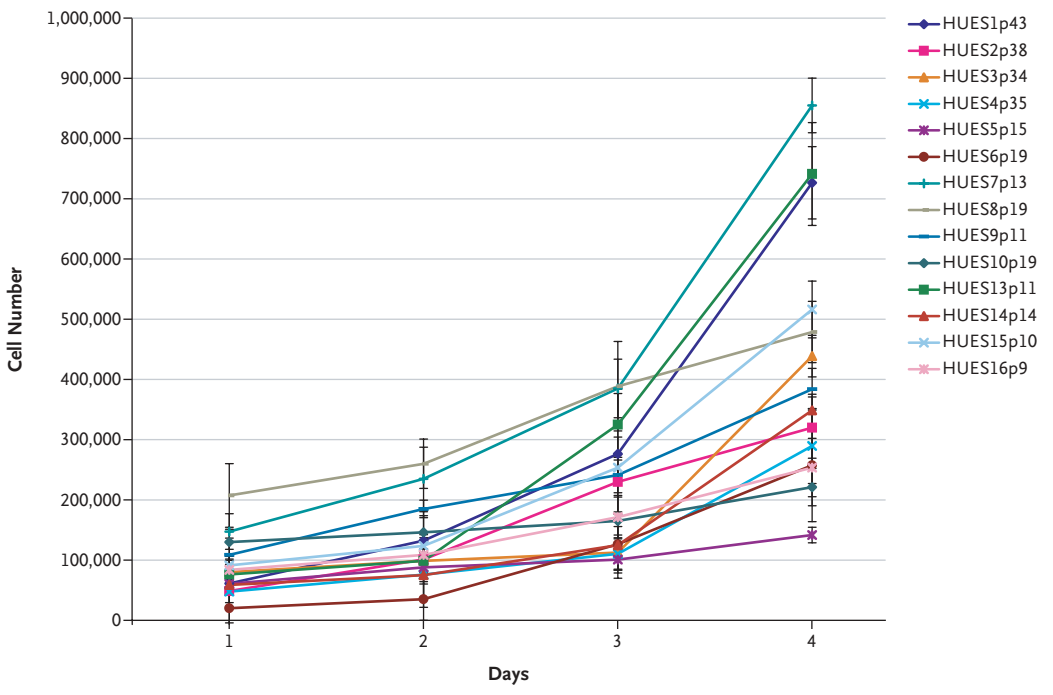
Supplementary Figure 1. Undifferentiated marker characterization.

Fluorescent immunostaining of cell line HUES17 with A) anti-Oct-4 antibodies (red), B) anti-SSEA3 antibodies (red), C) anti-SSEA4 antibodies (red), D) anti-TRA-1-60 antibodies (red), and E) anti-TRA-1-81 antibodies (red). F) Alkaline phosphatase activity (red) for cell line HUES17. In each panel nuclei are blue (DAPI). All images at 10x magnification.



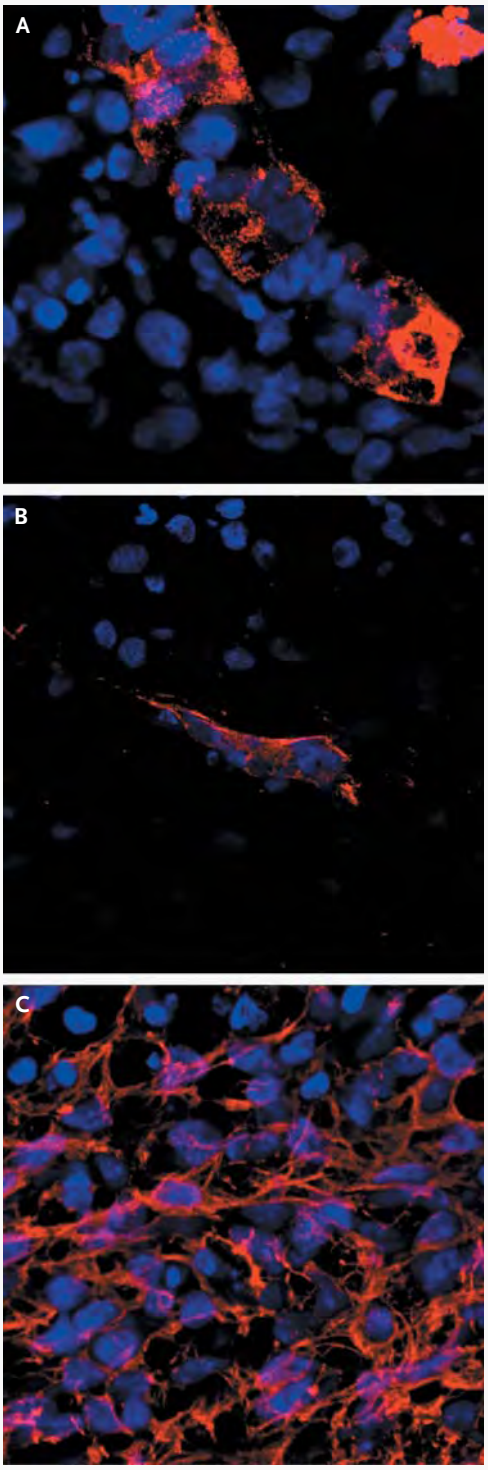
Supplementary Figure 2. Karyotype analysis of hES cell lines.

Shown are representative GTG-banded metaphase spreads of: A) HUES13 46,XY characteristic of a chromosomally normal male cell line and B) HUES5 46,XX characteristic of a chromosomally normal female cell line.



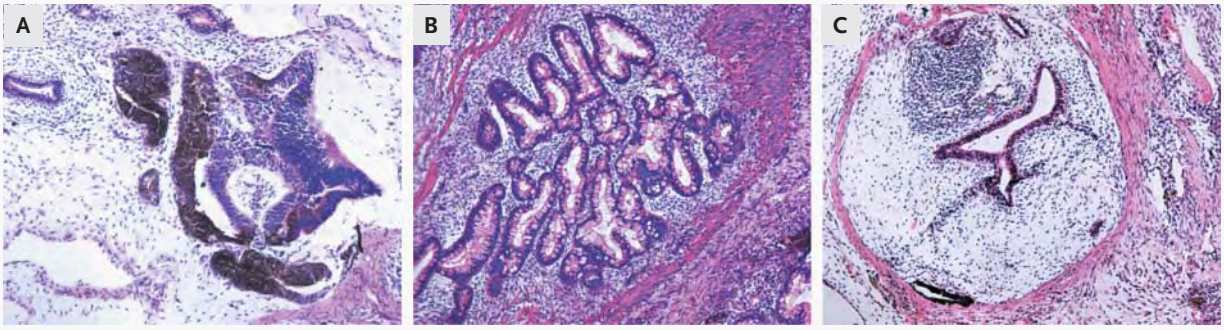
Supplementary Figure 3. Population doubling time of hES cell lines.

Cell counts for hES cell lines HUES1-10 and HUES13-16 are shown for each timepoint. The calculated population doubling times from these data are found in Table 1.



Supplementary Figure 4. In vitro differentiation of hES cell lines. Fluorescent immunostaining of cryosectioned EBs.

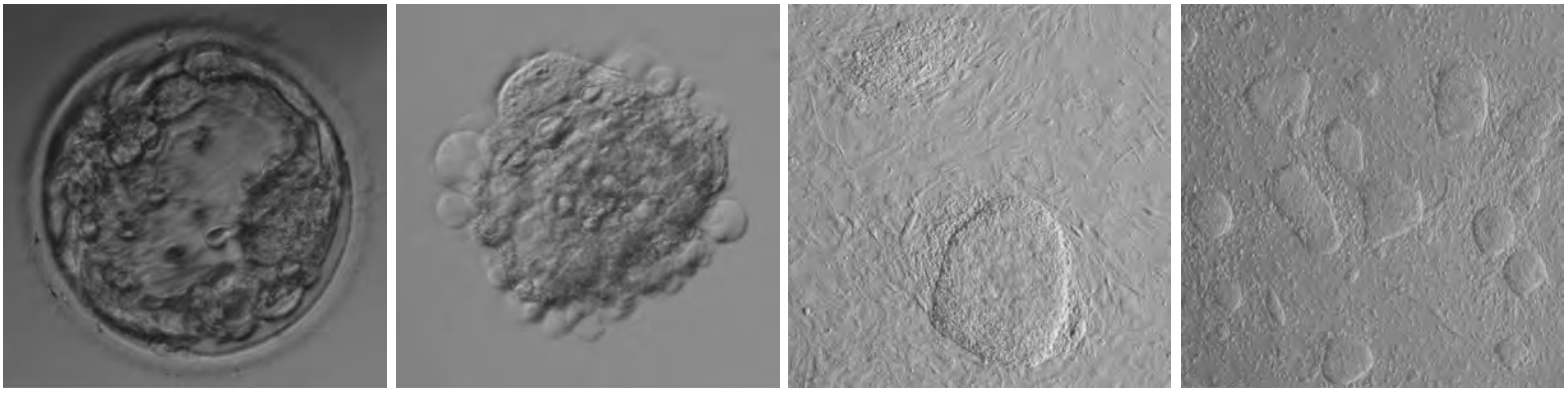
Immunostaining of cell line HUES6 with A) anti-AFP antibodies (red), B) anti-MF20 antibodies (red), and C) anti-Tuj1 antibodies (red). In each panel nuclei are blue (DAPI). Images are at 63x magnification.



Supplementary Figure 5. H & E stained images of teratomas.

Shown are representative H & E stained images of teratomas derived from HUES cell lines. A) HUES 1, B) HUES 2, and C) HUES 3 all at 10x magnification

Human Embryonic Stem (HUES) Cell Collection



Instruction Manual
Version 1.0

HUES (Human Embryonic Stem) Cell Collection

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Derivation of Embryonic Stem Cells from Human Blastocysts

Publication

Cowan, C.A. et. al, Derivation of Embryonic Stem Cells from Human Blastocysts (2004), NEJM in press.

Derivation

Supernumerary frozen human cleavage stage or blastocyst embryos produced by in vitro fertilization for clinical purposes were donated after informed consent and institutional review board approval. Embryos were cultured to the blastocyst stage and the zona pellucida removed by digestion with acid tyrodes, followed by immunosurgery using rabbit anti-human RBC antibodies and a guinea pig sera complement. (cover image depicts the derivation of HuES-12)

Characterization

The cell lines presented here are similar to other reported human embryonic stem (hES) cells with a high ratio of nucleus to cytoplasm, prominent nucleoli, and compact colony morphology. The hES cell lines were found strongly positive for a number of molecular markers of undifferentiated pluripotent human stem cells, including octamer binding protein-3/4 (Oct-3/4), stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. These results are consistent with the molecular characteristics reported for existing hES cell lines.

Expansion

17 of the human embryonic stem (HUES) Cell lines that were derived in our hES facility have been expanded to distribution numbers. With the exception of HUES-4, all lines were expanded before passage twenty two. A number of the lines were expanded before passage 15. All cell lines are verified to be viable after thaw and competent to grow and undergo enzymatic passaging, if using the protocol and reagents included in this instruction manual. The following table lists each line, its passage number, and karyotype[1].

Available lines

Cell Line	Passage	Karyotype
HUES-1	15	46,XX
HUES-2	16	46,XX
HUES-3	26	46,XY
HUES-4	21	46,XY
HUES-5	12	46,XX,inv9
HUES-6	17	46,XX
HUES-7	11	46,XY
HUES-8	21	46,XY
HUES-9	13	46,XX,inv9
HUES-10	10	46,XY
HUES-11	13	46,XY
HUES-12	13	46,XX
HUES-13	15	46,XY
HUES-14	16	46,XX
HUES-15	12	46,XX
HUES-16	15	46,XY
HUES-17	15	46,XY

[1] Routine karyotypic analysis of hES cell lines is warranted (see Maintenance and Passaging p.9)

Storage, Growth Conditions and Technique Notes

Shipping and Storage

hES cells and mitotically inactivated MEFs (PMEF*i*) have been supplied in 90% fetal calf serum and 10% dimethyl sulphoxide frozen stocks. Each hES vial contains ~ 4 x 10^5 cells and is sufficient for plating one 35 mm tissue culture treated plate (1 well of a 6- well plate). Each PMEF*i* vial contains ~4 x 10^6 cells and is sufficient for plating 6 wells of a 6-well plate. Vials are shipped on dry ice. Upon receipt, store hES vials in $N_2(l)$ and PMEF*i* vials at -80°C.

***Successful thaws and splits have been carried out on Specialty Media's hygromycin and neomycin resistant, pre-treated MEFs. (Not supplied)**

Growth/ Atmospheric Conditions

Ambient Temperature :	37 ± 0.5°C
CO_2 concentraion :	5.1 ± 0.6% CO_2
Relative Humidity :	85 – 100 %

General Technique

Tissue Culture

General tissue culture techniques should be observed when working with hES cells. All protocols should be carried out using sterile/aseptic technique in an appropriate tissue culture room and under a laminar flow hood. Additionally, our hES facility has taken an added precaution to reduce airborne particulate by installing air handling and filtration equipment to produce a pseudo-clean room. Gloves are worn when handling all reagents and material that come in contact with cells. (This includes opening of incubators.) All workspaces are thoroughly cleaned with 70 % isopropanol before and after use.

Media/Materials

All media and reagents are filtered sterilized prior to first use with a 0.2µm filter. Media bottles and TC materials should be sprayed down with 70% isopropanol before being placed in the hood.

Cell Handling

We recommend thawing no more than two samples at a given time to ensure for easy handling. The handler should take care not to leave cultures at room temperature and low CO_2 for long periods of time.

All centrifugation of live cells is done at 500-600 x *g* for 5 min at room temperature.

Methods, Media Preparation

Media preparation

Both MEF and hES media are prepared under sterile/aseptic conditions. After preparation, media is filter sterilized at 0.2 µm.

When preparing hES growth media, a general consistency will be important. It is advisable to work with the same lot numbers of reagents whenever possible. It is especially important to work with lots of serum replacement which meet the criterion presented in Appendix CAT- 1.

Growth factors should be the last reagents added to the media. It is important to note that bFGF requires a protein carrier when being re-suspended. DO NOT try to re-suspend bFGF in minimal media.

MEF media

MEF media	550 ml
DMEM	500 ml
PenStrep	5.5 ml
Gluta-MAX™	5.5 ml
FBS	55 ml

hES media

hES media	650ml
KO-DMEM	500ml
PenStrep	6.5 ml
Gluta-MAX™	6.5 ml
NEAA	6.5 ml
2-mercaptoethanol	0.65 ml
Serum Replacement	65 ml
Plasmanate	65 ml
hLIF (12 ng/mL final)	0.78 ml
bFGF[2] (~10 ng/mL final)	0.52 ml

Freezing media

Freezing media is a mixture of 90% fetal calf serum and 10% dimethyl sulphoxide. Filter sterilize at 0.2 µm and store at 4°C.

Gelatin preparation

Prepare a 0.1% solution of gelatin in milli-Q (tissue culure grade) water. Ensure that the gelatin is thoroughly dissolved by heating. Autoclave or filter sterilize the solution at 0.2 µm before use.

[2] Resuspend in 1mL DMEM plus 0.1% BSA.

Methods, Gelatin Coating and Preparation of MEF Plates

Gelatin Coating

Prior to plating any cells, coat tissue culture treated plates with 0.1% gelatin (prepared with milli-Q water) for a minimum of 30 minutes at 37°C. Use 1.5 ml or 4 ml gelatin solution to coat a 35 mm or 10 cm plate respectively. Aspirate off the gelatin solution just before thawing MEFs.

MEF Preparation

A reliable feeder layer of mouse embryonic fibroblasts (MEF) is important for the survival and growth of hES cells. With any HUES cell line, you will receive vials of pre-treated, mitotically inactivated MEFs (PMEF*i*), for the thaw and for one split. Each vial contains enough PMEFs (~4 x 10^6 cells) to cover one 6-well tissue culture treated plate. If extensive passaging/expanding is to take place, it is advisable to derive, expand and inactivate your own MEFs (see Appendix MEF-1 for details on deriving and expanding).

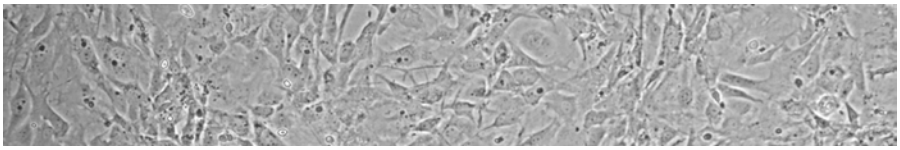
MEFs should be plated one to two days before thawing/passaging hES cells. **MEFs should not be used as a feeder layer for longer than 12-14 days.**

Plating MEFs

Pre-warm MEF media to 37°C. Aliquot 10ml of the media into a sterile 15 ml conical tube and set inside hood. Remove MEF vial from -80°C and immediately submerge the bottom half of tube in a 37°C water bath. It should take about 30-45 seconds before the cells are 80% thawed (small frozen portion left)[3]. Quickly bring the tube to the laminar flow hood, spray down with 70% isopropanol, and transfer cells to the 10 ml of pre-warmed media. It is recommended to wash the vial with an additional 1 ml of pre-warmed media (add to 15 ml conical).

Spin the 15 ml conical tube at 500-600 x *g* for 5 minutes. While cells are spinning, aspirate the gelatin solution from plates prepared earlier, and set inside hood. After spin is complete, carefully aspirate the media without disturbing the pellet. Re-suspend the pellet in 12 ml (6 well plate) pre-warmed MEF media. Aliquot 2 ml of MEF solution in a drop wise manner into each of the six wells. Be sure to distribute the MEFS evenly about the well. Date the MEF plate, and place in a 37°C incubator overnight to allow MEFs to attach to plate.

After 6 hours MEFs will be attached to the plate. MEFs should be confluent. It is best to use MEF plates 24-48 hours after plating. DO NOT use a MEF plate that is over 4 days old.



Gamma irradiated pretreated MEFs (PMEF*i*)--24 hours after plating (10x)

[3] Thaw time will vary with volume (based on 1 ml). Avoid thawing different volumes at the same time.

Methods, Thawing Human Embryonic Stem Cells

hES cells

The human embryonic stem cells you have received were frozen down at an ~1:6 split from a sub-confluent, 10 cm tissue culture dish. Most vials are frozen at a concentration of ~4 x 10^5 cells/ ml. HUES-9 is frozen in a volume of 0.5 ml and should be thawed by itself, as it will take less time than the other lines to thaw. It is recommended to thaw one vial into one 35 mm tissue culture dish, or one well of a 6-well plate.

Thawing/Plating hES cells
Part C

Before thawing, ensure that the MEF plate you have already prepared is properly plated and in good condition. DO NOT try to use a less than desirable plate, or one that is older than three days. It is recommended to pre-label all conical tubes and wells being used.

Pre-warm hES media to 37°C. Aliquot 10 ml hES media into a sterile and labeled 15 ml conical tube for each line. Remove hES vial/s from N_2(l) and immediately submerge the bottom half of the tube in a 37°C water bath. It should take about 45-60 seconds before the cells are 80% thawed (small frozen portion left)[4]. Quickly bring the tube to the laminar flow hood, spray down with 70% isopropanol, and gently transfer cells to the 10 ml of pre-warmed media. It is recommended to wash the vial with an additional 1 ml of warmed media (add to 15 ml conical).

Centrifuge the 15 ml conical tube at 500-600 x *g* for 5 minutes. While cells are spinning, remove pre-plated MEFs from incubator. Under the hood, aspirate off the media from as many wells as you will be thawing into. Quickly, aliquot 1 ml pre-warmed hES media back into each well of the plate, being careful not to disturb the attached MEFs. Set the plate aside in the hood. After spin is complete, carefully aspirate the media without disturbing the pellet (if necessary, do not aspirate all media). Gently re-suspend each pellet in 1 ml of pre-warmed hES media. Add the hES solution, in a drop wise manner, to a MEF plate well which already contains 1 ml hES media. As with MEFs, best results are obtained if the drops are evenly distributed about the plate. Carefully return the plate to a 37°C incubator overnight to allow the hES cells to seed the MEFs.

[4] Thawing hES cells will take slightly longer than MEFs due to colder storage temp (based on 1 ml).

Methods, Maintenance and Passaging Human Embryonic Stem Cells

hES cell maintenance

hES cell culture is demanding. With the exception thaw/split days, media should be changed every day. Additionally, a growth lag after a thaw or passage often occurs. For this reason, it is very important to observe the cells every day. Be sure to prepare MEF plates ~ 2 days in advance as splits may catch you by surprise.

Please note, we have observed karyotypic changes involving trisomy of chromosome 12 (in two cell lines; HUES-3 and 4) as well as other changes (additions to chromosome 2 in HUES-1) in the HUES cell lines. These karyotypic abnormalities are accompanied by a proliferative advantage and a noticeable shortening in the population doubling time. As chromosomal abnormalities may be commonplace in hES cell lines, we recommend frequent karyotypic analysis.

Day 1

24 hours after thawing the hES cell stocks, check the wells under 4x magnification. You may notice that there is quite a bit of debris, which is normal. Do not be alarmed. The debris is a mixture of dead cells. At this early stage, you should not expect to see many colonies. If the layer of dead cells completely covers the well, it is recommended to perform a partial media change.

Day 2-4

Typically, the first full media change takes place 48 hours after thawing the hES cells. 2 mls per well is recommended. Colonies may start to become visible as early as the second day post thaw. Continue changing the media every day.

Day 4-7

In our experience, cultures may require passaging as early as 4 days post-thaw and as late as 10 days post thaw (at this point the MEFs are old). The number of days before a split will depend on a number of factors including how well the thaw has been carried out. On average, expect that a culture will take seven days to become sub-confluent. For an example of how a given cell line performs, please contact us.

Do not be discouraged if your thaw does not recapitulate the images in the appendix. We have noticed that there is some inconsistency in thawing, and that some lines behave more reliably than others. It is not uncommon to see cultures which exhibit ragged, flat colonies, or in the extreme case, a monolayer of cells. DO NOT give up on your thaw. It is common for the cellular morphology of HUES cell lines to improve after one or two passages.

hES cell passaging

After 4-7 days post-thaw, your cells should be near confluence. A sub-confluent hES cell culture is generally split at a 1:3 ratio. It is important to split your cells before they differentiate. If the colonies begin to turn a brownish color prior to reaching sub-confluence, it may be necessary to split the cells early and at a 1:2 ratio. Likewise, the split may be adjusted if the cells are too dense (evenly distributed and touching one another).

Methods, Maintenance and Passaging Human Embryonic Stem Cells, Cont'd

1:3 split

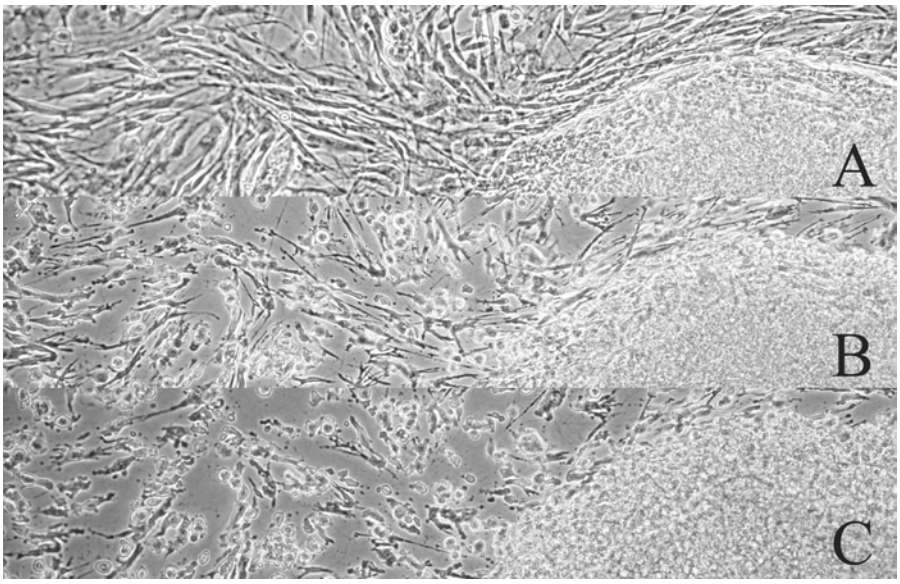
Before splitting, ensure that you have three wells of pre-plated MEFs. Allow MEFs to attach for at least 24 hours, but no more than three days.

Pre-warm hES media and 0.05% Trypsin/EDTA to 37°C. Under the hood, pre-label one sterile 15 ml conical tube. Remove MEF plate from incubator. Under the hood, aspirate off the media from three wells. Quickly, aliquot 1 ml pre-warmed hES media back into each well of the plate, being careful not to disturb the attached MEFs. Set the plate aside in the hood.

The following steps should be done quickly to minimize the amount of time that cells are not in hES media.

Carefully aspirate the hES media from the culture to be split. Gently wash the well with 1X phosphate buffered solution (PBS). Aspirate the PBS. Add 0.3 ml 0.05% Trypsin/EDTA to the well. Replace lid, and observe the cells under 4x magnification. The MEFs surrounding the hES colonies should begin to retract.

Trypsinization
Figure 1



When the MEFs are sufficiently rounded and the borders of the hES colonies are rough, return the plate to the hood. Add 2 ml pre-warmed hES media to the trypsinized cells. Gently pipette up and down, washing the bottom of the well, until the MEF monolayer has completely detached (monolayer is sticky and may remain in one piece). Transfer the cell suspension to the 10 ml conical tube. Wash the well one more time with an additional 1 ml hES media, transferring it to the same conical tube. At this point the trypsin should be adequately inactivated.

Pipette the solution 5-7 times with an automated pipette. Aliquot 1 ml of the hES solution drop wise, making sure to distribute the drops evenly about the well. Without shaking the plate, carefully return the cells to a 37°C incubator overnight to let hES colonies seed.

hES cells coming out of a split should behave similarly to those coming out of a thaw. The maintenance guidelines presented in the previous section can also be followed for splits.

MEF Derivation Protocol

Mouse preparation

Mouse embryos are harvested at 12.5 days post-coitum, from natural ICR matings.

Collection of embryos

Before collection, gelatin coat 150mm tissue culture treated plates (Part A). We prepare one plate per 1.5-2 embryos. Generally expect 12 embryos per mouse (6-8 plates). Additionally, prepare three non-tissue cultured 10cm plates per mouse with sterile 1X PBS.

Pre-warm MEF media and 0.05% Trypsin to 37°C.

Euthanize ICR females and remove uterus with embryos and place into 1X PBS. Under a microscope in a laminar flow hood remove embryos from their deciduas, dissect and discard the internal organs (intestines, liver, heart, etc.) and transfer the cleaned embryos to a dry, sterile 10cm Petri dish.

Using a sterile blade, mince the embryos. Add 10ml warm 0.05% trypsin per 10-14 embryos. Pipette up and down until the small pieces of embryo are homogenized. Transfer the solution to a 50 ml conical tube. Incubate at 37°C for 1 minute. Pipette the solution 5-10 more times.

Plating primary MEFs

Add 40 ml pre-warmed MEF media to solution. Spin for 10 minutes at 500-600 x *g*, at room temperature. Aspirate media and then re-suspend pellet with 30 ml pre-warmed MEF media. Plate 1.5-2 (~5 ml solution) embryos per 150 mm gelatin coated plate. The final volume of the plates should be 20 ml. Place plates in 37°C, 5% CO_2 incubator.

When plates reach confluence, split them 1:3 to 1:4 to a new tissue culture treated (non-gelatin coated) 150 mm plate. Incubate at 37°C and 5% CO_2 until cells are again confluent.

Freezing primary MEFs

Pre-warm MEF media and 0.05% trypsin to 37°C. Place freezing media (Media Preparation, p.7) on ice. Aliquot 20 ml pre-warmed MEF media into a 50 ml conical tube (1 tube:3 plates).

Remove plates, three at a time, from incubator. Aspirate media from plate. Wash once with 5 ml 1X PBS. Add 5 ml trypsin to each plate. Allow cells to detach. Transfer trypsin solution to 50 ml conical tube. With an additional 5 ml trypsin, wash all three plates, and add the solution to the 50 ml conical. Perform a final wash with 10 ml fresh, pre-warmed MEF media. Again, adding the solution to the 50 ml conical tube. Spin cells at 1000 x *g* for 5 min. Gently re-suspend pellet in 9 ml freezing media (1:3 split). Aliquot 1 ml (~3 x 10^6 cells) per labeled, cryogenic vial. Freeze at -1°C / minute[5]. Store in N_2(l).

[5] In the hES facility, vials are placed in styrofoam, at -80°C overnight.

MEF Expansion

MEF expansion

One vial of primary (passage 1) MEFs will expand to a 150 mm plate in ~4-7 days. Expect ~10 x 10^6 cells (~3, 6-well plates) for every expanded 150 mm plate.

Pre-warm MEF media to 37°C. Aliquot 10 mls MEF media into a 50 ml conical tube, per vial to be thawed. Remove vial/s from N_2(l) and immediately submerge the bottom half of the tube in a 37°C water bath. It should take about 45-60 seconds before the cells are 80% thawed (small frozen portion left). Quickly bring the tube to the laminar flow hood, spray down with 70 % isopropanol, and gently transfer cells to the pre-warmed media. Spin down the cells at 500-600 x *g*. Re-suspend pellet in 5 ml pre-warmed media per tube thawed. Prepare an appropriate number of 150 mm tissue culture treated plates by aliquoting 15 ml pre-warmed MEF media to each. Add 5 ml of MEF solution to each 150 mm plate, taking care to distribute cells evenly. Label plates (date, passage 2) and place in 37°C, 5% CO_2 incubator.

Treating MEFs

Once confluent, MEFs must be mitotically inactivated either by mitomycin C treatment, or by δ-irradiation.

Mitomycin C inactivation

For mitomycin C treatment of plates, remove 15 ml of culture media/plate and transfer to a 50 ml conical tube. Add an appropriate volume of mitomycin C such that the final concentration is 10 μg mitomycin C / ml culture media. Mix solution well. Aspirate remaining 5 ml of culture media from each plate, and add back 15 ml of the mitomycin C containing media. Incubate at 37°C, 5% CO_2 for three hours.

Pre-warm MEF media and 0.05% trypsin to 37°C. Aliquot 20 ml pre-warmed MEF media into a 50 ml conical tube (1 tube: 3 plates).

Remove plates, three at a time, from incubator. Aspirate media from plate. Wash once with 5 ml 1X PBS. Add 5 ml trypsin to each plate. Allow cells to detach from plate. Transfer solution to 50 ml conical tube. Wash all three plates with 5 ml trypsin, add solution to 50 ml conical tube. Perform a final wash with 10 ml fresh, pre-warmed MEF media. Add solution to 50 ml conical tube. Count cells with a hemocytometer. Spin cells down at 500-600 x *g*. (continue on to plating or freezing)

δ-irradiation

An alternative to mitomycin C treatment is δ-irradiation. Prior to irradiation, trypsinize the MEFs as described above. Spin down, and re-suspend in a volume of MEF media that will be accommodated by the δ-irradiator. Irradiate the MEFs for 25 minutes at 247.3 Rads/min for a total exposure of 6182.5 Rads. More than 25 minutes at room temperature and normal atmospheric conditions is not recommended. After irradiation, increase the volume of the MEF solution to 50 ml. Count cells with a hemocytometer. Spin cells down at 500-600 x *g*.

Plating fresh-treated MEFs

If there is an immediate demand for inactivated MEFs, re-suspend pellet in pre-warmed MEF media to a concentration of ~4 x 10^6 cells/ 12 ml if plating a 6-well plate (~4 x 10^6 cells/ 10 ml for a 10 cm). Follow directions for plating MEFs (p. 8)

Freezing pre-treated MEFs

If there is no immediate need for treated MEFs (PMEF(*i*)), they can be frozen at a concentration of ~4 x 10^6 cell/vial. Freeze in freezing media (Media Preparation, p.6) at a rate of -1°C/ minute. Store at -80°C.

Materials and Reagents

Materials / Catalogue no.

KO-DMEM	Invitrogen Gibco	CAT#10829
DMEM	Invitrogen Gibco	CAT#11965-092
FBS	Valley Biomedical, Inc.	CAT#BS-3033
*KO-Serum Replacement	Invitrogen Gibco	CAT#10828
Plasmanate	Bayer	CAT#NDC 0026-0613-20
bFGF	Invitrogen Gibco	CAT#13256-029
hLIF	Chemicon International	CAT#LIF1010
2-beta-mercaptoethanol	Gibco	CAT#21985-023
Non-essential amino acids (NEAA) (10mM or 100x)	Invitrogen Gibco	CAT#11140050
Pen/Strep (5000U/ml Penicillin G sodium and 5000ug/ml Streptomycin sulfate in 0.85% saline)	Invitrogen Gibco	CAT#15070-063
Glutamax-I (200mM or 100x)	Invitrogen Gibco	CAT#35050-061
Mitomycin C	Sigma	CAT#M-0503
0.05% Trypsin/EDTA	Invitrogen Gibco	CAT#25300-054
Dimethyl sulphoxide (DMSO)	Sigma	CAT#D2650
Gelatin	Sigma	CAT#G1890
Tissue Culture Treated Plates (6 well)	Corning	CAT#3471
Hygromycin resistant, pre-treated MEFs	Specialty Media	CAT#PMEF-H
Neomycin resistant, pre-treated MEFs	Specialty Media	CAT#PMEF-N

* Our facility uses KO-Serum Replacement with an endotoxin level < 1.0 EU/mL and osmolarity > 460 mOsm/kg.