Materials Submitted to NIH
From Advanced Cell Technology (ACT)
Submission #2010-ACD-007

I. Submission Cover Page p. 1
II. Section IIB Assurance p. 3
III. Research Consent p. 4
IV. IVF Clinical Consent p. 9
V. Cryopreservation Consent p. 10
VI. Protocol p. 12
VII. Additional Information p. 26

NOTE: Duplicative information in the submission is not included.
### hESC Registry Application Search Results

<table>
<thead>
<tr>
<th>Request #:</th>
<th>2010-ACD-007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Status:</td>
<td>Pending</td>
</tr>
<tr>
<td>Review:</td>
<td>ACD</td>
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<tr>
<td>Assurance:</td>
<td>Yes (Section II(B))</td>
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<tr>
<td>Certification:</td>
<td>Yes</td>
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<td>Cell Lines:</td>
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</tr>
<tr>
<td>Available:</td>
<td>1</td>
</tr>
</tbody>
</table>
| Previous #: | 2010-DRAFT-005  
             | 2010-ADM-006 |

**Organization:** Advanced Cell Technology, Inc.  
**Org Address:** P.O. Box 1700 Santa Monica, CA 90406-1700  
**DUNS:** 021958041  
**Grant Number(s):**  
**Signing Official (SO):** William Caldwell / 310-576-0611 / wcaldwell@advancedcell.com  
**Submitter of Request:** Kathy Singh / 508-756-1212 ext 653 / kssingh@advancedcell.com  
**Submitter Comments:** (None)

**Line #1:** MA135  
**NIH Approval #:**  
**Available: Yes**  
**Embryo from U.S.: Yes**  
**Embryo Donated in Year(s):**  
**Provider Name:** Advanced Cell Technology, Inc.  
**Provider Phone:** 508-756-1212 ext 653  
**Provider Email:** kssingh@advancedcell.com  
**Provider URL:**  
**Provider Restrictions:** For research purposes only.

**NIH Restrictions:**

**Additional Information:**

**Supporting Documents:**  
**Document 1:** (PDF - 02/19/2010) Consent - Elements: 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15  
**Document 2:** (PDF - 02/19/2010) Assurance Letter (Caldwell) - Elements: 16

**Administrative Comments:** SO certifications updated 26 Feb 2010  
IIB certification uploaded 13 April 2010  
10 May 2010 email uploaded 11 May 2010 by Diane Hannemann  
Consent for IVF uploaded 11 May 2010 by Diane Hannemann  
Derivation Protocol uploaded 11 May 2010 by Diane Hannemann  
Blastocyst Grading for Transfer uploaded 11 May 2010 by Diane Hannemann  
Consent for Cryopreservation uploaded 11 May 2010 by Diane Hannemann  
Transfer of Frozen Embryos uploaded 11 May 2010 by Diane Hannemann
NIH Staff IIB Analysis uploaded 17 May 2010 by Diane Hannemann

Administrative Attachments:
Document 1: (PDF - 02/26/2010) re. definition of hesc
Document 2: (PDF - 03/23/2010) NIH senior staff concur moving to ACD
Document 3: (PDF - 04/13/2010) Section IIB assurance
Document 4: (PDF - 04/13/2010) annotated consent (sent with IIB assurance)
Document 5: (PDF - 05/11/2010) 10 May email
Document 8: (PDF - 05/11/2010) Blastocyst Grading for Transfer
Document 11: (DOC - 05/17/2010) NIH Staff IIB Analysis

Status History:
Draft: 02/19/2010
Pending: 02/19/2010

Emails Sent: 02/19/2010 New Application Email

Previous ADM Request Number: 2010-ADM-006
Switched from ADM to ACD Date: 03/23/2010
Reason for Switch to ACD Review:
Submission was reviewed under Section IIA and found not to meet those criteria.

Added By: Commons_william_caldwell On: 02/19/2010 | Last Updated By: NIH_hannemann On: 05/17/2010 | Record ID: 51

Total Record Count = 1

http://hescregapp.od.nih.gov/login/list.htm?DetailList=yes&id=51

5/18/2010
March 24, 2010

Ellen Gadbois, Ph.D.
National Institutes of Health
Office of Science Policy Analysis
Bldg 1 Room 218D

Dr. Gadbois,

I hereby assure that the embryo from which cell line MA135 was derived, was donated prior to July 7, 2009, and the embryo: 1) was created using in vitro fertilization for reproductive purposes and was no longer needed for this purpose; and 2) was donated by individuals who sought reproductive treatment ("donor(s)") who gave voluntary written consent for the human embryo to be used for research purposes. I understand that the Working Group of the Advisory Committee to the NIH Director will consider submitted materials taking into account the principles articulated in Section II(A) of the NIH Guidelines for Human for Human Stem Cell Research, 45 CFR 46 Subpart A, and the following points to consider: during the informed consent process, including written and oral communications, whether the donor(s) were: (1) informed of other available options pertaining to the use of the embryo; (2) not offered any inducements for the donation of the embryo; and (3) informed about what would happen to the embryo after the donation for research.

For clarity, I attach the consent used for MA135 and have highlighted and notated the sections that address the specifics of this assurance.

(A) Embryo was donated prior to July 7, 2009
(B) Embryo was created using in vitro fertilization for reproductive purposes and was no longer needed for this purpose
(C) Embryo was donated by individuals who sought reproductive treatment ("donor(s)") who gave voluntary written consent for the human embryo to be used for research purposes
(D) Donors were informed of other available options pertaining to the use of the embryo
(E) Donors were not offered any inducements for the donation of the embryo
(F) Donors were informed about what would happen to the embryo after the donation for research
(G) Donors received written and oral communications regarding the consent.

Thank your for your review of our application. I am available should you have any questions.

Respectfully,

William Caldwell IV
Chief Executive Officer
Consent to Donation of Excess Embryos
For Stem Cell Research

INSTRUCTIONS:

This form is for use by couples who have undergone IVF procedures to create
embryos for their reproductive purposes, using their own gametes (ova and sperm).
In order to donate embryos for use in stem cell research, both gamete providers
must be available and willing to sign the consent form.

You are being provided this document because you have undergone fertility treatment at
________________________ (name, address and telephone number of IVF Clinic),
which has stored embryos created by you and your partner for your future reproductive
use. You and your partner have determined that some or all of these embryos are no
longer needed for your reproductive purposes and have indicated that you may be willing
to donate these excess embryos to Advanced Cell Technology for use in research. This
document describes the research, including its risks and benefits (if any). It also contains
other information that you should consider before agreeing to donate your embryos.

Before deciding to donate your embryos, you may also wish to read the attached
information sheets put together by the National Institutes of Health. The information
sheets contain general information about stem cell research. If you are interested in the
specific stem cell research that is being carried out by Advanced Cell Technology, you
can check the company's website at http://www.advancedcell.com.

I. PURPOSES OF STEM CELL RESEARCH:

One purpose of stem cell research is to study ways in which to generate stem cells.
Another purpose is to study how stem cells might be used to treat certain diseases. Stem
cells are cells that may (if research goes well) repair the body by re-growing damaged
organs or tissues. Understanding stem cells is a very active area of scientific research at
this time because there are many possible uses for them. For example, when brain cells
die, as in Parkinson's disease, or heart cells die as a result of a heart attack, replacement
cells are not available. Likewise, diabetes results from the loss and non-replacement of
special cells that make insulin. Research on animals suggests it may be possible to
develop human stem cells for use in treating such diseases, and to restore normal
function. For the research to go forward, a source of human stem cells is needed. An
excellent source of such cells may be frozen human embryos that are in excess of those
needed for reproductive purposes (see Subheading III. Procedure).
II. PROCEDURES INVOLVING DONATED EMBRYOS:

If you agree to donation, the IVF Clinic will transfer one or more frozen embryos belonging to you and your partner to Advanced Cell Technology, a company whose main office is in Worcester, Massachusetts. Advanced Cell Technology will thaw and culture each embryo in one of its laboratories for up to six days, usually until it reaches the blastocyst stage (about a hundred cells). The resulting blastocyst will be used to produce a line of immortalized, pluripotent human stem cells (that is, cells capable of continuing to multiply for an unlimited period of time in the laboratory and becoming virtually any tissue in the human body). The donated embryo or embryos will be destroyed as a result of the process by which stem cells are derived. At no time will your donated embryo(s) be implanted in a woman’s uterus.

Advanced Cell Technology may keep donated embryos in a frozen state for a period of weeks or months. However, once an embryo is thawed and allowed to begin development, the embryo will be destroyed as part of the research process before it reaches the age of fourteen days, the time when the first development of organs begins.

III. RESEARCH USES OF STEM CELLS:

Once human stem cells have been derived from donated embryos, Advanced Cell Technology will use the stem cells to study the process of cell development, and to try to make cells and tissues that can be transplanted into humans to treat various diseases. Such research might involve genetic manipulation of the cells or the mixing of human and nonhuman cells in animal models. Advanced Cell Technology has an independent Ethics Advisory Board that will review all future research uses of stem cells. In addition, an institutional Review Board will review any future research involving transplantation of stem cells into human beings.

Since stem cells are capable of continuing to multiply for an unlimited period of time, Advanced Cell Technology may keep stem cells that have been derived from your embryos for many years.

IV. RISKS OF DONATION:

Because these embryos have already been created for your reproductive purposes, donating your embryos for research poses no additional medical risks to you or your partner. However, following your donation, it is possible that one or both of you might experience psychological discomfort with your decision to donate your embryos for research purposes, and this discomfort could require counseling support.

In the unlikely event of a breach of confidentiality (see below, item X), you may also be exposed to unwanted publicity.
ADVANCED CELL TECHNOLOGY

V. BENEFITS OF DONATION:

There will be no benefits to you as a result of your embryo donation to Advanced Cell Technology. You cannot designate the stem cell lines resulting from this research for the medical benefit of any particular individual. However, by donating your excess embryos for stem cell research, you may help researchers make important advances in medical knowledge which could be of benefit to others in the future.

VI. COMPENSATION:

You will not receive any compensation for your donation. However, once you have signed this consent form to donate your excess embryos for research, you will not be responsible for any subsequent costs associated with storing the donated embryos. The IVF Clinic will bill Advanced Cell Technology directly for any storage costs incurred after the date of your donation.

VII. ALTERNATIVES:

You can choose not to donate an embryo or embryos to Advanced Cell Technology. Possible alternatives include indefinite continued storage, donation of embryos to other infertile couples, or removal from storage with disposal. If you choose not to donate, this will have no effect upon any future care provided to you by the IVF Clinic where the embryos are stored.

VIII. WITHDRAWAL:

If you decide to donate and later change your mind, you can withdraw consent up until the time the embryos are actually removed from storage at the IVF Clinic for transfer to Advanced Cell Technology. The time period between the signing of the consent form and the transfer of the embryos to Advanced Cell Technology is on average two weeks. Notice of withdrawal must be received by the IVF Clinic before the embryos are transferred to Advanced Cell Technology in order for withdrawal to be effective. Notice of withdrawal should be sent to the IVF Clinic in writing, at the address shown on the first page of this form.

IX. COMMERCIAL DEVELOPMENT:

As a commercial firm, Advanced Cell Technology hopes to develop new therapies and products from this research that will be financially profitable. The cells, products, research results and any intellectual property directly or indirectly developed from research using your embryos may have commercial value to Advanced Cell Technology. You will not receive any financial benefits from such future commercial developments.
ADVANCED CELL TECHNOLOGY

X.  CONFIDENTIALITY:

Prior to transferring your embryos to Advanced Cell Technology, all information associated with the embryos that might enable Advanced Cell Technology to readily identify you or your partner (such as name, address, or social security number) will be removed and replaced with a code. The key to the code, which key provides the link between you and the embryos you donate to Advanced Cell Technology, will be retained at the IVF Clinic. The IVF Clinic will retain the key for as long as any of your frozen embryos, or cell lines created from your embryos, remain in existence, which could be for an indefinite period of time. The IVF Clinic will not release this key to Advanced Cell Technology under any circumstances.

As your identity will be unknown to Advanced Cell Technology, you will not be identified in any publications or public statements issued by Advanced Cell Technology about its research.

At some point in the future it is possible that stem cell lines produced from your embryos will be used in the treatment of other individuals. If that happens, the U.S. Department of Health and Human Services or the Food and Drug Administration may need to review the coded research records maintained by Advanced Cell Technology, together with the records maintained by the IVF Center that contain the key to the code. The records maintained by Advanced Cell Technology and the IVF Center might also be reviewed or audited by others, who will be bound by the same confidentiality rules that prevent disclosure of your identity to Advanced Cell Technology.

XI.  CONSENT TO DONATE EMBRYOS FOR PURPOSES OF STEM CELL RESEARCH

We, ______________________ and _____________________, have read all materials presented to us and have had them explained to us by ____________________.

We understand all of the following:

Donation of our embryos will be of no direct benefit to either one of us or to anyone we know;

The embryo(s) donated by us will be used to produce human stem cell lines for research on cell replacement or cell transplant therapies;

Each donated embryo will be destroyed by the stem cell derivation process and no embryo will be transferred to a woman's uterus to produce a baby. No donated embryo will be allowed to develop beyond thirteen days. However, any stem cells produced may be kept for many years;

The cells, products, research results and any intellectual property directly or indirectly developed from research using our embryo(s) may have commercial value to Advanced
ADVANCED CELL TECHNOLOGY

Cell Technology but we will not receive any financial benefits from any such future commercial developments;

The research findings from our donated embryos may be described and reported in scientific journals and meetings. Our identities will not be revealed in any such reports and we will not personally benefit in any way from such reports;

Our decision to donate embryos for this research study is purely voluntary.

We have read all of the above, including the description of the purposes of stem cell research and the research procedures involving donated embryos. We have asked questions concerning any areas we did not understand, and we have received satisfactory answers to these questions. We willingly give our consent to donation of our excess embryos to Advanced Cell Technology for the research purposes described above. We wish this consent to supersede any document we may have signed in the past regarding the disposition of our embryos.

Name of Female ___________________________ Signature ___________________________ Date ________

Name of Male Partner ___________________________ Signature ___________________________ Date ________

Witness Name ___________________________ Signature ___________________________ Date ________

Name of Person Conducting Informed Consent Discussion ___________________________ Signature ___________________________ Date ________

XII. CERTIFICATION:

I certify that my ova were used to create the embryos that I wish to donate to Advanced Cell Technology. I further certify that I am not an employee of Advanced Cell Technology.

Name of Female ___________________________ Signature ___________________________ Date ________

I certify that my sperm was used to create the embryos that I wish to donate to Advanced Cell Technology. I further certify that I am not an employee of Advanced Cell Technology.

Name of Male Partner ___________________________ Signature ___________________________ Date ________
CONSENT FOR IN VITRO FERTILIZATION (IVF)

We, the undersigned, acknowledge that we have chosen to participate in the process of In Vitro Fertilization at

We have read the documents listed below. The use of medications and procedures listed below have been described to us verbally and in writing by the physicians and staff of ; and all of our questions have been answered. We consent to the procedures that are medically indicated for our care, and we assume the associated responsibilities and risks.

Overview of In Vitro Fertilization ( )
IVF Hurdles
Multiple Gestation and Maternal Pregnancy Reduction ( )
Side effects of Gonadotropins ( )
Intracytoplasmic Sperm Injection (ICSI)
Assisted Hatching ( )
Stimulation Culture and Transfer ( )

We agree that our acceptance into treatment and continued participation in an IVF cycle is at the sole discretion of the physicians. We understand that pregnancy is in no way guaranteed, and that should a pregnancy result, we will assume all risk associated with that pregnancy, such as multiple gestations and/or other complications of pregnancy and delivery.

We give consent to contact any physicians who have provided care to us before, during and after a pregnancy. We understand that information regarding our cycle outcome will be sent to the Society of American Reproductive Technology (SART), and to the Center for Disease Control (CDC), in accordance with the law.

We give consent to have information shared with both partners at any time during the cycle. We agree to our permission to have messages left at the telephone numbers provided by you to the

We understand that a responsible adult must be available to take us home after undergoing procedures, and remain with us for the next 24 hours. If both partners are having procedures, the responsible adult giving the ride home must be present at the clinic before the procedures will begin.

We agree to be financially responsible for any medical expenses not covered by insurance.

(Partner-print) 12-14-06 (Data)
(Partner-print) 12-14-08 (Data)
(Witness-print) 1/14/05 (Signature) (Date)

12/11/05
EMBRYO CRYOPRESERVATION CONSENT

We have read and discussed the information presented concerning embryo cryopreservation and have been given the opportunity to ask questions. We understand the information and accept the risks, either known or unknown, of freezing embryos. We understand that all reasonable efforts are made by the

in the event of embryo death. We understand that mechanical problems could occur that may result in the premature thawing and loss of viability of these embryos. There is no guarantee that any embryos will survive thawing and subsequent culture or that a pregnancy will result from this procedure. We further understand that even if a child is born, there exists the possibility that he/she or they may in some way be abnormal. We release the

and parents from any and all liability concerning any aspects of this procedure.

We request cryopreservation of any normal developing embryos.

In the case of the death of any of us, we have chosen to:

A. / Donate the embryos anonymously to another infertile person(s).
B. / Donate the embryos to the for research.
C. / Donate the embryos to be used for Stem Cell Research.
D. / Dispose of the embryos according to the current acceptable guidelines.

Printed/Handwritten Name: __________________________

Date: ________________ 80 - specify

Witness: __________________________

Date: ________________ 80 - specify

If signing this form outside of ________________ must be notarized

I certify that I know or have satisfactory evidence that:

Before the person (s) who appeared before me, and said person (s) acknowledged that he/she/they signed this instrument and acknowledged it to be free from any imposed or involuntary act for the uses and purposes mentioned in the instrument.

SUBSCRIBED AND SWORN TO: Before me this day of ________________ 20__

Signed: __________________________ Notary Public in and for the State of
Residing in __________________________ Commission Expires: __________________________
1. I hereby authorize Dr. [name], and/or such associates, assistants, or designees, including medical residents in training, to select the following condition(s) which have been explained to me: [name], resident, or designee(s). The nature of the condition(s) is explained in professional and lay language.

2. The procedures planned for treatment of my condition(s) have been explained to me by my physician. I understand them to be: [describe procedure to be performed in professional and lay language.]

3. I recognize that, during the course of the operation, post operative care, medical treatment, anesthesia, or other procedures, unforeseen conditions that may require additional or different procedures than those above may be encountered. I authorize my attending physician, and his or her designee(s), to perform such surgical or other procedures as are in the best interests of my health and are not within my control at the time the medical or surgical procedure is commenced.

4. I have been informed by my physician that there are significant risks such as: [list risks] which may result in partial or total disability, which may be attendant to and a consequence of each operation. I acknowledge that no warranty or guarantee has been made to me as to results or cure.

PHYSICIAN'S STATEMENT: The medical procedure or surgery stated on this form, including the possible risks, complications, alternative treatments, including non-treatment and anticipated results, was explained to me in professional and lay language before the patient or his/her representative co-signed.

PHYSICIAN'S SIGNATURE:

DATE: 12/14/05 TIME: 10:00

PATIENT OR PATIENT REPRESENTATIVE'S ACKNOWLEDGMENT: I acknowledge that I have read (or have had read to me) and fully understand the above consent, the explanation referred to above made to such patient or his/her representative before the patient or his/her representative co-signed.

PATIENT SIGNATURE:

DATE: 12/14/05 TIME: 09:40

WITNESS ACKNOWLEDGMENT: I acknowledge that I, as witness, have identified the above individual and have observed his/her signature on this document.

WITNESS SIGNATURE:

DATE: 12/14/05 TIME: 09:40

NAME PLATE:

[Signature]
Approaches for Derivation and Maintenance of Human ES Cells: Detailed Procedures and Alternatives

Irina Klimanskaya and Jill McMahon

Introduction

Deriving human embryonic stem (hES) cells is a challenging endeavor. Although derivation of mouse ES cell lines has become a common procedure, the limited number of currently available hES cell lines is testament to the difficulties encountered at various stages of their derivation and maintenance. The common techniques for maintenance of hES cells involving mechanical passaging of the cells using collagenase or dispase are often complicated and introduce variability in the growth potential of the hES cells. Distributors of the available hES cell lines frequently recommend attending special training courses prior to working with the hES cell lines they provide.

In our lab, we recently established and characterized 17 hES cell lines, described in Cowan et al. (2004). These lines have been adapted to trypsinization, which simplifies the passaging of the cells and generates cells in sufficient numbers to permit experimentation. All of these lines can be successfully frozen and thawed using very simple procedures; with a recovery rate of 10% or higher.

Techniques for deriving and maintaining pluripotent human and mouse ES cells in culture have been described by a variety of labs, and there are notable similarities and differences. For the derivation and maintenance of hES cell lines in our lab, we adapted previously published methods and developed an approach that consistently produced new cell lines and that proved to be easily taught to other investigators.

This chapter describes the aspects of derivation and maintenance of hES cells that we found to be helpful in generating hES cell lines including the equipment used, preparation and quality control of media and reagents, cell passaging techniques, and other aspects of hES cell morphology and behavior.

Setting Up the Lab

EQUIPMENT

Initial steps in the derivation process are conducted under a dissecting microscope. We make an effort to keep embryos and dishes containing the early mechanically passaged dispersions at 37°C. Dishes brought out of the incubator are set on 37°C slide warmers or viewed using microscopes fitted with heated stages. The mechanical dispersions necessitate having the dishes open for extended periods. Since the cultures are vulnerable to contamination during this time, we have the dissecting microscope within a bench-top laminar flow hood. A high-quality stereo microscope with a wide-range zoom is essential for the mechanical dispersion of colonies, including the inner cell mass (ICM) outgrowth; it permits an overall assessment of each plate and evaluation of the morphology of each colony when doing the mechanical passaging.

The equipment used in our lab is as follows:

Stereomicroscope for microdissection: A Nikon SMZ-1500 with the magnification range 10–100x works well with its easy zoom and the positioning mirror that regulates the depth and contrast of the image. A whole 35-mm dish can be scanned for colony morphology, and the zoom permits selection with precision of the parts of the colony that are the best for dissection.

Inverted microscope: A Nikon TE 300, or any regular inverted cell culture microscope, set up with phase and Hoffman modulation contrast (HMC) optics with phase objectives of 4x, 10x, 20x, and HMC 20x and 40x. HMC is recommended for viewing ES cells and is required for embryö evaluation.

Heated microscope stage for both stereo and inverted microscope. A (Nikon) slide warmer keeps extra PME plates at 37°C during mechanical dispersions.

Bench-top laminar flow hood with a HEPA filter: Vertical hoods by Terra Universal (Anaheim, California). Horizontal models sometimes produce too much vibration. We found that these vertical hoods were tall enough to accommodate a dissecting microscope and were very convenient and reliable.

Tissue culture incubator: All parameters (CO2 concentration, humidity, and temperature) need to be checked daily with external monitoring equipment.

External monitoring equipment:
• Surface thermometer
• Mercury liquid-immersed thermometer
38. Approaches for Derivation and Maintenance of Human ES Cells: Detailed Procedures and Alternatives

- Hygromycin
- CO₂ monitor and gas calibration kit (BD 444 series by CEA Instruments, Emerson, New Jersey), calibrated regularly

QUALITY ASSURANCE OF EQUIPMENT
Consistency in growth conditions is very important for the development of the embryos and the growth of the hES cells. The checklist of parameters monitored daily includes the percentage of CO₂ (5.0), temperature (37°C), and humidity of the incubators (>90%). A checklist with daily readings is very helpful for timely recalibration if an undesirable trend is noticed. Warming rings and platforms are constantly monitored with surface thermometers.

Two incubators are set aside for derivations and expansion of new lines. The incubators are checked prior to any new derivation round by growing mouse embryos from the two-cell stage to blastocyst; a passing score requires 50% to go to blastocyst. Cultures at early stages, prior to being frozen, are split between the two incubators as a protection against incubator failure. The incubators are not opened frequently, thereby maintaining steady growth parameters.

STERILITY
Some aspects of the derivation of hES cell lines put the associated work under more stringent sterility requirements than those of any typical cell culture lab. These include the limited availability of frozen human embryos, the labor-intensive nature of derivation and expansion, the team effort involved, and the long periods in culture until the newly established lines can be expanded and safely frozen. In addition, because hES cells are prone to spontaneous differentiation under unfavorable conditions, many labs prefer not to use antibiotics in cell culture media.

The reagents should either be purchased sterile from the manufacturer or be filter-sterilized in the lab. Most of the cell culture supplies can be bought sterile. However, everything that is sterilized in-house by autoclaving or by dry heat needs to be quality controlled with biological indicators (spore strips from Sterile, Mentor, Ohio).

If it comes to the worst, the triple-action drug Norocin (see Media Components) appears to be tolerated by hES cells without significant changes in their pluripotency or growth rate and permits the rescue of contaminated cultures.

Preparing and Screening Reagents
The hES cell lines in our facility were derived and continue to be grown on primary mouse embryo fibroblast (PMEF) monolayers. We derived the lines in media containing Serum Replacement and Plasmamate, a component of the medium used in the IVF field for thawing human embryos and fetal bovine serum (FBS) at early stages of derivation.

MEDIA COMPONENTS
- KO-DMEM (Invitrogen, Cat. No. 10829)
- DMEM high glucose (Invitrogen, Cat. No. 11960-044)
- Serum Replacement (Invitrogen, Cat. No. 10828): Each lot needs to be tested, but as a guide, we found that the lots with osmolality higher than 470 mOsm/kg and endotoxins lower than 0.9 EU/ml were the best. Upon thawing, make single-use aliquots and freeze.
- Plasmamate (Bayer, Cat. No. 613-25): Each lot needs to be tested.
- FBS (Hyclone, Cat. No. SH30070.02): Each lot needs to be tested. Heat inactivate, if desired, and freeze in aliquots.
- β-mercaptoethanol, 55 mM (100x) solution (Invitrogen, Cat. No. 21985-023)
- Non-essential amino acids (NEAA), 100x solution (Invitrogen, Cat. No. 11400050)
- Penicillin-streptomycin, 100x solution (Invitrogen, Cat. No. 15070-063)
- Glutamax-I, 100x solution, a stable dipeptide of L-glutamine and L-α-phospho-L-lysine, a glutamine substitute (Invitrogen, Cat. No. 35050-061)

Penicillin-streptomycin and Glutamax-I are kept in frozen single-use aliquots.

- Basic fibroblast growth factor (bFGF) (Invitrogen, Cat. No. #13256-029): Add 1.25 μl of hES cell growth media without leukemia inhibitory factor (LIF) or bFGF to a vial containing 10 μg of bFGF. This makes an 8 μg/ml stock solution. Increasing final bFGF concentration to 8-20 μg/ml can be beneficial for the cells, especially at early stages of derivation, after thawing or when the cells are grown at low density. Make 120-μl aliquots and freeze.
- Human LIF (Chemicon International, Cat. No. LIF1010)
- 0.05% trypsin-0.03 mM EDTA (Invitrogen, Cat. No. 25300-054)
- Gelatin from porcine skin (Sigma, Cat. No. G1880)
- Phosphate-buffered saline (FBS), Ca²⁺, Mg²⁺-free (Invitrogen, Cat. No. 14190-144)
- Normocin, an antibiotic active against gram +/− bacteria that also has antimycoplasmal and antifungi activity (Invitrogen, San Diego, California; Cat. No. ast-mr-2, comes as a 500x solution)

MEDIA RECIPES
Bottles of media that are opened frequently become alkaline rapidly; we suggest making smaller quantities that will last approximately a week.

PMEF Growth Medium
To a 500-ml bottle of high-glucose DMEM, add:
- 6-ml penicillin-streptomycin
- 6-ml Glutamax-I
- 50-ml FBS
- hES Cell Basal Medium
To a 500-ml bottle of KO-DMEM, add:
- 6-ml penicillin-streptomycin
- 6-ml Glutamax-I
- 6-ml NEAA
- 0.6-ml β-mercaptoethanol
Preparing PMEF Feeders

ES Cell Derivation Medium

Use this medium at early stages of ICM outgrowth. It has low LIF and bFGF concentration and contains FBS. You can switch to hES cell growth medium when a steady growth of colonies has been reached (usually passage 2–4).

1. Add 100 ml of basal medium, add:
   - 10 ml Plasmáate
   - 10 ml Serum Replacement
   - 40 µl of human LIF (final concentration 20 ng/ml)
   - 20 µl of bFGF stock solution (final concentration 8 ng/ml)
   - More (up to 20 ng/ml)
   - Sterilize by 0.22-µm filtration

2. hES Cell Growth Medium

200 ml of basal medium, add:
   - 10 ml Plasmáate
   - 10 ml Serum Replacement
   - 160 µl of human LIF for 10 ng/ml, or 480 µl for 20 ng/ml
   - 20 µl of bFGF stock solution (final concentration 4 ng/ml)
   - 1x bFGF, or more if a higher concentration is desired
   - Sterilize by 0.22-µm filtration

Gelatin

Dissolve 0.5 g of gelatin in 500 ml of warm (50-60°C) Milli-Q water. Cool to room temperature, and sterilize by 0.22-µm filtration. This makes 0.1% solution.

Mitomycin C

Add 1 ml of sterile Milli-Q water to a vial (2 mg) of purified mitomycin C (Sigma, Cat. No. M 0503); this makes 1 mg/ml stock solution. The solution is light sensitive and is good for one week at 4°C.

SCREENING MEDIA COMPONENTS

It is important to be consistent with screening, aliquoting, and storage of the media components. Various lots of Serum Replacement, Plasmáate, and FBS should be screened, preferably on hES cells. The screening of Serum Replacement lots should be done prior to the first lot running out so that an evaluation of its qualities can be compared side by side to the previous lot. Some newly derived hES cells will die out with a change in lots at the initial stages.

Screening of FBS, Plasmáate, or Serum Replacement

This test is based on a published procedure for screening FBS lots for mouse ES cell work according to Robertson (1987). This approach can be used for screening any combination of reagents or for finding the best concentrations for media supplements. The quality of the reagents is assessed by counting the number of colonies, evaluating the morphology of the hES cells, and staining for alkaline phosphatase activity as detected with Vector Red Kit (Vector Laboratories, Burlingame, California).

1. Prepare 12-well plates with PMEFs. For each lot tested, you will need at least 12 wells to vary the concentration of the component being tested from the working concentration to high enough concentrations to evaluate toxicity: 8%, 10%, 20%, and 30%. Each concentration is done in triplicate and compared to media components at working concentrations known to support hES cell growth.

2. Split hES cells onto 12-well plates with a ratio of 1:6–1:10. The difference in reagents will be more noticeable when the cells are started at low density. However, some cell lines grown very slowly and differentiate when they are kept at a low density. Therefore, adjust the splitting ratio to the specific hES cell line. Resuspend the cells in a small volume of basal medium and add equal volumes of cell suspension to each well of the test plate. Pipette up and down in each well or slowly move the plate in perpendicular directions for even distribution of the cells. Do not rotate because doing so will move most of the freshly added cells to center.

3. Change the medium daily, and evaluate colony morphology under the microscope. Human ES cells grow in flat, tightly packed colonies with sharp circular borders. The colonies appear deep red when stained for alkaline phosphatase activity. In differentiating colonies, the cells are more loosely packed, with definite borders, and stain pinker. Usually, the difference in the conditions being tested becomes more obvious as colonies grow bigger. However, as the colonies grow larger, they can begin to touch each other, and they tend to differentiate. Staining one of the triplicate wells for alkaline phosphatase activity prior to seeing signs of differentiation is advisable. Continue with the other wells in each set for another day or two before staining (see Figure 38-1 for a sample test).

Adequate recordkeeping for all commercial and in-house prepared lots of reagents is helpful for troubleshooting should the hES cells begin to exhibit differentiated morphology.

Preparing PMEF Feeders

We grow our hES cells on PMEF feeders that have been mitomycin C treated to generate stable monolayers. The PMEFs are made by standard procedures using 12.5 days postcoitum (dpc) (ICR) mouse embryos as described in Robertson (1987). The 12.5dpc embryos are Ectodermically, but the heads are left on during tissue disruption in trypsin; plating density is 1.5 embryos per 150-mm plate. PMEFs are expanded once after the initial plating (1:5 split) and then frozen (P1); the growth rate of PMEFs and their performance as feeders decrease as they go through multiple passages; therefore, thawed PMEFs are only passed once (P2) for expansion purposes prior to mitomycin C treatment, at which point a new vial of PMEFs would be thawed.

MITOMYCIN C TREATMENT AND PLATING

Mitomycin C is added to the media of a confluent plate of PMEFs at a concentration of 10 µg/ml and incubated at 37°C for three hours. The cells are harvested by trypsinization and placed on gelatinized plates in PMEF growth medium. In
serum-free hES cell growth media, the PMEPs may appear less confluent because of the spindle-like shape the cells take on. To ensure a confluent monolayer, we recommend a plating density of 30,000 to 60,000 cells/cm². We prefer to use plates of PMEPs no longer than 3–4 days after mitomycin-C treatment.

Mechanical Passaging of hES Cell Colonies

Many established hES cell lines are passaged with collagenase or by trypsin in conjunction with mechanical dispersion. Mechanical dispersion can provide colonies of “perfect” morphology, as it can be done to selectively pick undifferentiated colonies or even undifferentiated parts from differentiated and overgrown colonies, but it is time consuming and does not yield large numbers of cells; this limiting expansion of the hES cell lines. Nevertheless, this procedure is invaluable at early stages of derivation or as a means of producing more homogeneously undifferentiated plates of cells for expansion or for adaptation to trypan blue. It is also a tool for a “rescue operation” in critical situations when the success of salvaging a few colonies means saving an hES cell line.

MATERIALS NEEDED

Flame-Pulled Thin Capillaries
We use an alcohol or gas burner, to pull presterilized glass Pasteur pipettes into finely drawn capillaries. The capillaries are broken by hand into angled tips, the shape of a hypodermic needle. The diameter of the capillaries may vary, but the best results are achieved when they are 10 to 100 ES cells in diameter; this is how large the colony pieces are going to be. The choice of diameter depends on the operation. For instance, to do initial dispersion of an ICM outgrowth or to target undifferentiated parts of a colony, a diameter of 10 to 30 cells would be used.

Mouth-Controlled Suction Device
Similar to a mouth pipette used for embryo transfer, this device consists of a mouthpiece (Meditech International, Cat. No. 1501 P), rubber tubing, and a 0.22-μm syringe filter with a rubber tubing adapter for the Pasteur pipette. It provides precision in all manipulations for colony dispersions.

MECHANICAL DISPERSION
The procedure is similar to vacuuming. Gentle dispersion of the colony is achieved by simultaneously cutting off the pieces with the angled end of the capillary, very lightly moving them off, and sucking them in. With the opening of the capillary positioned nearly horizontal to the bottom of the dish, begin moving from the sides toward the colony center, chopping off and gently sucking in each piece. The light suction helps detach colony pieces and is applied at all times as you move from the periphery of the colony, collecting the colony parts. If the whole colony is coming from the monolayer in one piece, it is probably differentiated and should be discarded.

When the desired number of colonies is dispersed, blow out the pieces into the same plate for ICM dispersion or into a freshly prepared plate (see later sections of this chapter for details about preparing the receiving plate). To avoid having all the colonies stick to each other in the center of the plate, move the plate gently from side to side; do not swirl. (Figure 58-2 shows examples of mechanically passaged cultures.)
Derivation of hES Cells

Many factors that influence whether an isolated ICM will produce a hES cell line are not fully understood. Some of the factors to consider are what stage the embryo was frozen at and by what procedure; the length of time the embryo must be in culture to generate a blastocyst, the culture conditions, and the quality of both the ICM and the trophectoderm. When an embryo is ready for immunosurgery, it must be determined empirically, usually occurring between day 3 and day 7. Any embryo that has undergone cavitation and has a relatively intact trophectoderm is a candidate for immunosurgery.

IMMUNOSURGERY

The process of immunosurgery was performed essentially as described by Solter and Knowles (1975). It involves removing the zona pellucida with Acidic Tyrode’s solution, incubating the embryo in an antibody that binds to the trophectoderm and preferably not to the ICM cells (especially important for the embryos with nonintact trophectoderm), and then lysing the trophectoderm cells with complement. The dead cells that surround the ICM are removed by suctioning the ICM through a narrow capillary. The isolated ICM is put on a prepared PMEF for further growth and dispersion.

Materials needed:
- Acidic Tyrode’s (Specialty Media, Cat. No. MR-004.D)
- Rabbit anti-human RBC antibody (purified IgG fraction, Inter-Cell Technologies, Hopewell, New Jersey, Cat. No. AG 28840): Aliquotted and stored at -80°C; freshly diluted 1:10 in hES cell derivation medium
- Complement (Sigma, Cat. No. S1639): Aliquotted and stored at -80°C; freshly diluted 1:10 in derivation medium
- Capillaries for embryo transfer: Thinly drawn capillaries

Prepared Mitomycin C-Treated PMEF Plates

For the initial ICM outgrowth, change the medium on a four-well plate of mitomycin C-treated PMEFs to hES cell derivation medium the night before the immunosurgery to let it get conditioned by the PMEFs, final volume of 250 µl.

Instead of conditioning it overnight, the derivation medium can be supplemented with 30% of an hES cell-conditioned medium. To collect an hES cell-conditioned medium, add medium to a near-confluent culture of hES cells with good morphology. (see Figure 38-2A for an example of colony density and morphology), leave for 24 hours, collect the medium, filter, and store for 2–3 days at 4°C.

IMMUNOSURGERY PROCEDURE

1. Each embryo is processed separately. A dish is prepared with a series of 30-µl microdrops, three for each step: Acidic Tyrode’s, anti-human RBC antibody, complement, and three drops of derivation medium for each wash. The drops are covered with embryo-tested mineral oil and are equilibrated in the CO2 incubator for 60 minutes.
2. Under the dissecting microscope, transfer the embryo into the first Acidic Tyrode’s drop for a quick (1–2 seconds) wash, then move into the second drop. Watch the embryo
closely; as soon as the zona pellucida thins and is nearly dissolved, move the embryo into the next series of hES cell medium drops. Move the embryos through the first antibody drop into the second and third drops; then put the dish into the incubator for 30 minutes.

3. Transfer the embryos through three drops of derivation medium and through three drops of the complement solution as described previously; incubate in the last drop of complement for 15 minutes and check for any "bubbling" trophoblast cells. If no cells show signs of lysis, or if only a few cells are bubbling, continue the incubation and recheck in five minutes. The embryo should be transferred to the drop of differentiation medium as soon as all trophoblast cells are lysed or if no new bubbling cells appear after rechecking; the total incubation in complement should not exceed 30 minutes.

4. Gently pass the embryo through the opening of a thin, drawn pipette (about the diameter of the ICM); the lysed trophoblast cells should detach after 1–2 passes.

5. Wash the ICM in the drops with derivation medium and place into the prepared well of a four-well plate. The ICM should attach within 24 hours.

ICM DISPERSION

At early stages of derivation, we recommend doing the first dispersion as soon as at least 2–3 colony pieces can be obtained from the initial outgrowth (Figure 38-3). The dispersed colonies may be left in the same well or moved to a new well. If there are only a few colony pieces (1–5), they should be placed close to each other but with enough space to permit growth. It is better to disperse colonies before they grow into contact with each other and prior to signs of differentiation, such as becoming multilayered.

When the colony growth is slow, change two-thirds of the medium every 2–3 days to keep it conditioned at all times. As more colonies appear, change two-thirds of the medium daily and increase volume to 500 μl/well of a four-well plate.

Even if an original colony looks differentiated or comes off as a single piece, when replated; it usually gives an outgrowth of hES cells. When doing the initial dispersion, part of the original colony should be left untouched as a backup; especially if the picked pieces are transferred into a new well. Expect it to grow back in 1–2 days; the new outgrowth can be picked and recombined with previously picked cells. Multiple harvests can be obtained from the initial outgrowth. It is critical at this stage to expand the number of colonies slowly and steadily. See Figure 38-4 as an example of the length of time between dispersions and appearance of the cultures during the process of derivation. In this case, no immuno- surgery was done because the trophoblast was not sufficiently intact.
Maintenance of Established hES Cell Cultures

Usually, once steady growth of colonies is reached, use of the hES cell derivation medium is discontinued and the cultures are maintained in hES cell growth medium. For established cultures, remove one-third of the medium from growing culture, put it on a new PMEM dish, and add two-thirds volume of fresh medium. Change two-thirds of the medium daily; the medium should not turn yellow. Cultures should be expanded gradually by the progression from relatively sparsely populated four-well dishes to confluent four-well dishes to 35-mm dishes. Throughout the process, the cultures should be observed daily, differentiated colonies should be removed, and undifferentiated colonies should be dispersed as necessary (see Figure 38-5 for example approaches to be used for dispersion of colonies with different morphologies).

By the time the cells are growing on a 35-mm dish or a six-well plate, it is usually sufficient to disperse 50 to 100 average-sized colonies to populate a new well. In 1 to 2 days, it may be necessary to disperse some of the larger colonies, leaving the pieces in the same well. Usually, mechanical passaging needs to be done every 5 to 6 days, but several larger colonies may need to be dispersed daily.

ADAPTATION OF hES CELLS TO TRYPsin

Our experience with trypsinization of 20 newly established by us hES cell lines as well as of H1, H7, and H9 hES cell lines described in Thomson et al. (1998), demonstrates that after the initial adaptation of the lines to trypsin, this procedure can be robust and yield large quantities of hES cells that exhibit all the properties of pluripotent cells. Trypsinized cells retain undifferentiated colony morphology, express characteristic molecular markers (i.e., Oct-4, alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), differentiate into three germ layers in vitro and in teratomas, and maintain normal karyotypes.

Newly derived hES cells may be successfully passaged with trypsin as early as passage 2–3 from a four-well plate. However, trypsinization is not always successful, and several attempts may be necessary before the cells are adapted to trypsin; always keep a backup well of mechanically passaged cells.

The safest approach is to begin with a subconfluent 35-mm well of a six-well plate of colonies with good morphology.
38. Approaches for Derivation and Maintenance of Human ES Cells: Detailed Procedures and Alternatives

Figure 38.5. Approaches for dispersion of colonies with different methodologies. Various morphologies of hES cell colonies encountered at early stages of derivation when mechanical dispersion can be the tool of choice used to selectively pick undifferentiated colony parts. (A) All colonies are undifferentiated and can be mechanically passaged. (B) The colony that has few signs of differentiation (arrow) and is surrounded by differentiated cells, the undifferentiated part is easily separated from surrounding differentiated cells. (C) Partially differentiated, multilayered colonies like these are thickened and yellowish in color can be mechanically dispersed into several small pieces but may result in both differentiated and undifferentiated colonies. (D–F) All these colonies are more extensively differentiated: a thin layer of differentiated cells covers them like a veil. They can be cut into pieces through the top cell layer and passaged, and they may yield undifferentiated colonies. (G–I) These colonies are highly differentiated, the arrow on panel G shows a group of undifferentiated cells within the large differentiated area. If this one must be saved, wait a few days for this group to increase in size.

Figure 38.6. Adapation of mechanically passed hES cells to trypsin. (A) After five days of growth, colonies were mechanically dispersed and transferred to a fresh plate of SW56. (B) Morphologically, they are similar to the original plate. (C) The remaining colonies of the original plate, which were passaged with trypsin and plated onto a plate of the same growth area, show actively growing colonies and will probably be ready for passaging in 1–2 days.

Mechanically pick 50 to 100 colonies and transfer them into a new well as a backup. Differentiated colonies may be removed mechanically prior to trypsinization. Trypsinize the remaining colonies in the original well and plate into the same diameter well. The cells should be ready for the next split in 5 to 7 days (Figure 38.6). For the second trypsinization, split 1:3. After this step, the cells can usually be trypsinized routinely without problems, but a mechanical backup always should be maintained until the cells are frozen and the test vials are successfully thawed.

Trypsinization

Generally, hES cells recover from trypsinization better when they are not dispersed to single cells but remain as small
Maintenance of Established hES Cell Cultures

Lumps of approximately 2 to 20 cells. The procedure works best when hES cell colonies are dispersed by a combination of enzymatic digestion and pipetting; we do the pipetting before the PMEF monolayer, and the colonies turn into a single-cell suspension. The time in trypsin required for the cells to detach varies depending on the hES cell density, degree of differentiation, age of the culture, temperature of trypsin, and so on. Therefore, instead of providing a fixed incubation time in trypsin, we recommend checking the appearance of the hES culture under the microscope and empirically working out the best incubation time for each plate (Figure 38-7).

1. Warm the trypsin in a 37°C water bath; keep it warm until ready for the procedure.
2. Rinse the cells with PBS two times (1–2 ml per 35-mm dish).
3. Add 1 ml of trypsin to each 35-mm dish. Incubate in the hood at room temperature for several minutes, usually 2 to 5 minutes, frequently checking the cells under the microscope. The cells are ready for mechanical dispersion when the PMEFs begin to shrink; the colonies should round up but remain attached. Some cells may begin to detach and float (Figures 38-7A and 38-7B).
4. Prepare a centrifuge tube with 10 ml of warm PMEF medium.

Note: It is necessary to use PMEF medium to inactivate the trypsin because our hES cell medium is serum free.

Tilt the plate and begin to gently pipette the trypsin solution up and down with an automatic 1-ml pipette (Gilson type), pouring it over the cell monolayer at an angle. Properly digested cells should detach easily, leaving visible clear gaps in the monolayer where the trypsin solution was poured. If no such gaps appear, leave it for another 1 to 2 minutes and test again. Expect the monolayer to detach after several repetitions. On cell cultures less than 5 days old, you should be able to completely disperse the monolayer, but if the culture is older or very dense, there may be some undigested material that can be discarded. Usually, it takes 5 to 10 pipetting
strokes to break the colonies into small clumps of cells (see Figure 38-7C for the approximate clump size). Extensive pipetting should be avoided.

5. Transfer the trypsinized cell suspension into the prepared centrifuge tube; centrifuge for 5 minutes at 150 g.

6. Aspirate the medium, and resuspend the pellet in hES cell medium, again avoiding extensive pipetting to preserve small cell aggregates and to replate at the desired ratio. The colonies should become visible in 1 to 2 days, depending on the splitting ratio and the clump size (Figure 38-7D).

Human ES cultures passaged with trypsin can be maintained in an undifferentiated state. However, if conditions are unfavorable because of changes in media quality, a splitting ratio that is too high or low, or problems with PMEF quality, the cultures can have a degree of differentiation that should be evaluated prior to the next trypsinization (Figure 38-8).

Freezing hES Cells

Many of the established hES cells have low recovery rates upon thawing, as low as 0.1 to 1%. This may be because of the method of passaging the cells. Mechanical picking or using collagenase dispersion usually results in large cell aggregates, which presumably do not get cryopreserved as efficiently as smaller clumps. Trypsinized cells in our lab have a recovery rate of about 10 to 20%, or higher, and do not require more complicated procedures such as vitrification (Figure 38-9).

FREEZING MEDIUM

The best recovery rate was observed in freezing medium consisting of 90% FBS-10% dimethyl sulfoxide (DMSO). However, Oct-4 expression in the thawed cells was lower than in cells frozen in hES cell growth medium with 10% DMSO. Nevertheless, by the next passage, the expression and distribution of Oct-4 and other markers of undifferentiated cells were indistinguishable between these two freezing conditions. We routinely use the 90% FBS-10% DMSO medium.
hES Cell Quality Control

2. Make a thawing medium. We use 70% hES cell growth medium supplemented with 2x hLIF and 8 ng/ml bFGF with 30% hES cell- or PMEF-conditioned medium.

3. Change the medium on the PMEF plate to the thawing medium; equilibrate in the CO2 incubator for one hour. For 35-mm plates, use 1.5 ml medium; for four-well plates, use 0.5 ml medium per well.

4. Prepare a 50-ml conical tube with 10 to 15 ml of warm hES cell growth medium.

THAWING

1. Thaw the vial in a 37°C water bath, constantly agitating while ensuring that the neck of the vial is above the water level. Check the content of the vial after about 40 seconds and at 10-second intervals until only a small piece of ice remains.

2. Quickly spray the vial with 70% isopropanol, then using a 1-ml pipettor, add warm hES cell medium to the contents of the vial dropwise with gentle agitation. Do it quickly but very gently. Immediately transfer the contents into the prepared 50-ml tube with warm hES cell medium; centrifuge at 160 g for 5 minutes.

3. Remove the medium completely without touching the pellet.

4. Add 0.5 ml of hES cell thawing medium, gently resuspend the cells using a 1-ml pipettor (2 to 4 repetitions), and transfer to prepared PMEF plates with equilibrated hES cell-thawing medium. Spread the cells evenly throughout the well by moving the plate several times in two directions at 90 degrees to each other; avoid swirling.

5. Check the cells the next day; if there are many dead cells or the medium has changed color, change two-thirds of the medium; otherwise, do not change it for another day.

6. The colonies usually begin to appear in 3 to 4 days and can be ready for splitting in 5 to 10 days (Figure 38-7).

hES Cell Quality Control

Although the morphology of hES cells is often used for evaluating the quality of the culture and its readiness for passaging or freezing, this criterion alone cannot be used for an assessment of ES cell pluripotency. Staining for the expression of Oct-4 or alkaline phosphatase even in colonies of "perfect" morphology can result in one or both of these markers appearing in the cells only at the periphery of the colony. It is important, therefore, to regularly assess the cells by analyzing the expression of markers of pluripotent cells. We look at Oct-4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 by immunostaining, or we perform an enzyme assay for alkaline phosphatase. The procedures for such assays and available antibodies are described elsewhere.

KEY WORDS

Conditioned medium  Medium left in contact with cultured cells, usually for a prolonged period of time.

Thawing hES Cells

Thawing hES cells is a relatively simple procedure. The main rule to follow is to do everything quickly.

PREPARATION

1. Prepare mitomycin C-treated PMEFs a day before thawing.
Immunosurgery  A method of removing the trophoderm of a blastocyst using antibodies bound to the surface antigens of the trophoderm and complement.

PAIEP (primary mouse embryonic fibroblasts)  A mixed population of cells derived from dispersed mouse embryos and cultured for a limited number of passages under conditions favoring the growth of fibroblasts.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the Howard Hughes Medical Institution and the guidance and encouragement of Dr. Douglas Melton, the recipient of this HHMI grant, without whom this work could not have been accomplished. We would also like to thank our collaborators at Boston IVF, especially Dr. Jeannine Win nery, for contributing their invaluable expertise in the culture and evaluation of early embryos. A special thank you to Jocelyn Atienza and Chad Cowan for their ongoing work in developing and characterizing the hES cell lines reported on in this chapter. We are grateful to Drs. Martin Pera and Susan Lanzendorf for sharing helpful tips on the derivation and maintenance of hES cells. Lastly, we would like to thank Dr. Andy McMahon for his generous support throughout this project.

FURTHER READING


**Blastocyst Grading for Transfer**

*Purpose:* Assessment of day 5 embryos for uterine transfer. Use sterile technique throughout this procedure.

**Supplies Required**
- Stripping Pipets (MidAtlantic MXL3-275)
- The Stripper (MidAtlantic MXL3-STR)

**Embryo Assessment**
1. Remove culture dish from patient’s incubator and verify patient name and MR #.
2. Using a stripper pipet, move embryos into assessment drops, one embryo per drop.
3. Using 400X power on either ICSI scopes, record the degree of expansion, grade the inner cell mass and trophoderm using the following system.

**Degree of Expansion**
- EB1: The blastocoeel filling less than half of the volume of the embryo
- EB2: The blastocoeel filling greater that half of the volume of the embryo
- 3: The blastocoeel filling the entire embryo with little to no expansion of the overall size.
- 4: Fully expanded blastocyst
- 5: Hatching blastocyst
- 6: Fully hatched blastocyst

**Inner Cell Mass Grading**
- A: Tightly packed, compacted cells
- B: Larger, loose cells
- C: Small ICM
- D: No distinguishable ICM

**Trophoderm Grading**
- A: Many healthy cells forming a cohesive epithelium
- B: Fewer cells
- C: Very few cells, larger in size
Transfer of Frozen Embryos or Gametes

Purpose: To properly and safely transport human gametes or embryos. Report any unusual or abnormal events immediately to the supervisor or director and record in the lab diary.

Supplies Required
- Dry Shipper
- LN2 pan

A. Shipping

I. Charging the Shipper
Day prior to use of shipper:
1. Fill the shipper with liquid nitrogen.
2. Wait ten minutes and fill shipper again.
3. Repeat the filling process until absorbent is saturated and LN2 remains in canister.
4. Let sit overnight.
Day of use:
1. Fill the shipper with LN2 to verify the absorbent is saturated.
2. Before loading the shipper, pour off remaining LN2 (ship only in vapor phase — no liquid).

II. Transferring Embryos/Gametes to Shipper
1. Fill black lab pan with liquid nitrogen to a depth of 1-2 inches.
2. Remove gametes/embryos from storage tank and immediately plunge into LN2 filled pan.
3. Verify name and MR # on every vial or straw to be sent. Two embryologists must ID the samples.
4. Verify that the data on the cryo transport sheet matches the name, number of specimens, etc. to be sent. Embryos resulting from donor gametes must be labeled with a blue tab on the cryo cane.
5. Remove specimens from black pan and immediately place into canister of transporter. Remember to use a sleeve to cover vials on canes or second test tube to enclose straws.
6. Close shipper and lock.
7. Place shipper in shipping container along with paperwork. Include a copy of the patient’s datasheets and the thaw protocol. Make sure the datasheets have been FDA labeled and, if donor gametes were used, that a copy of the FDA eligibility sheet(s) is attached. Also include a copy of our FDA Specimen Labeling information sheet.
From: Kathy Singh [mailto:KSingh@advancedcell.com]
Sent: Monday, May 10, 2010 11:09 AM
To: Gadoit, Ellen (NIH/OD) [E]
Subject: FW: New HESC Registry Application Request #2010-ADM-006

From: Kathy Singh
Sent: Thursday, May 06, 2010 6:09 PM
To:
Subject: FW: New HESC Registry Application Request #2010-ADM-006

Dear Dr. Gadoit,
I have been able to obtain the information from the donating clinic you've requested below for MA135. I'm sorry for the delay in response; the clinic records needed to be retrieved from their archived files, so this took some time. Please see my answers inserted in blue below.

Thank you reviewing this. Please let me know if you have any further questions.

Kathy Singh

From: HESCREGISTRY (NIH/NIDCD) [mailto:hescregistry@mail.nih.gov]
Sent: Thursday, April 22, 2010 1:18 PM
To: William Caldwell; Kathy.Singh
Cc: HESCREGISTRY (NIH/NIDCD)
Subject: RE: New HESC Registry Application Request #2010-ADM-006

Hello Mr. Caldwell and Ms. Singh,

Thank you again for this submission. The ACD Working Group has conducted a preliminary review of this submission and has the following questions. Please let me know if you are able to obtain this information and documentation; if not available, please provide whatever information you can.

1. A general description of the arrangements through which the embryo used to derive MA135 was donated, including the following information:

- Whether the IVF clinic(s) at which the embryo(s) was created was separate from or affiliated with ACT.
The clinic was separate from ACT. ACT has no affiliations with any IVF clinics other than through a relationship we have developed with a few clinics to receive their donated embryos. In most cases these clinics have reached out to us as a source to fulfill the desires of their patients wishing to donate their embryos for research.

- Whether the embryo(s) was poor quality, remaining after PGD testing, or clinical grade embryo(s), and the procedures used for identifying those embryo(s) and shipping them to ACT.
The embryos for this donation were excellent (A) quality. We follow this general process for all embryo donations: The clinic is provided our IRB approved consent, which they then give to the patients who have expressed a desire to donate embryos they no longer need for their reproductive purposes. The patient is given an opportunity to have her questions answered regarding donation. After the consent is signed, the clinic contacts us and we arrange shipment. We provide a tank charged with liquid nitrogen via overnight courier and the clinic uses that tank to transport the embryos back to us. They ship to us the frozen embryos and associated consents from which they (the IVF clinic) have redacted all identifying information; they replace the name with a code for identification. (In this case the name was replaced with the code "Stem Cell 10"). Please see the clinic's two attachments, "Blissilacyst Grading for Transfer" and "Transfer of Frozen Embryos".

2. Copies of the consents for clinical care signed by the donors of the embryo(s) (with patient names redacted).
Please see the clinic's two attachments, "consent for IVF" and "consent for cryopreservation".

3. Specific dates of treatment and embryo donation.
I have been informed by the donating clinic of these three dates: patient started her IVF cycle on 12/20/05; IVF clinic froze the embryos on 12/20/05; embryos were donated on 1/11/06.

4. A copy of the research protocols.
See attached "Derivation Protocol" (which we wrote and still follow).

5. A description of any ethics review that was conducted regarding donation of the embryo(s) for research. Please also address
whether ACT or the donating IVF clinics are required to follow 45 CFR 46.
ACT's embryo donation consent was rigorously and thoroughly reviewed and approved by our IRB in January and March of 2008. The consent was previously approved as well by our EAB which was chaired by Ron Green who is the Director of Dartmouth's Ethics Institute and also included member Carol Tauer, who in 1994 was a member of the NIH Human Embryo Research Panel that was charged to make ethical recommendations for federal funding of research on infertility, pre-implantation diagnosis, and stem cell research. In 1999 she was a member of the NIH Working Group on Pluripotent Stem Cell Research that developed the specific ethical recommendations for federally funded human embryonic stem cell research. Neither ACT nor the donating clinic was required to follow 45 CFR 46.

Thank you again and please let me know if you have questions regarding this request.

Sincerely,

Ellen Gadbois

Ellen T. Gadbois, Ph.D.
Office of Science Policy Analysis
Bldg 1 Room 218D
National Institutes of Health
voice: 301-594-2567
fax: 301-402-0280

From: Gadbois, Ellen (NIH/OD) [E]
Sent: Tuesday, March 23, 2010 4:22 PM
To: wcaldwel@advancedcell.com; ksingh@advancedcell.com
Cc: HESCREGISTRY (NIH/NICHD)
Subject: RE: New hESC Registry Application Request #2010-ADM-006

Hello Mr. Caldwell and Ms. Singh,

We have reviewed this submission under Section IIA of the NIH Guidelines and have found that this submission does not meet all of the requirements under that section. However, it is eligible for review under Section IIB of the Guidelines. Therefore I am forwarding this submission for analysis by the Working Group on Human Embryonic Stem Cell Eligibility Review under Section IIB. Your submission still appears as pending review on the NIH public website.

For our records, could you please send me an assurance in accordance with Section IIB of the Guidelines? Essentially we need a statement from you attesting to the following:

I hereby assure that the embryo from which the cell line(s) identified in item 6 of the form was derived was donated prior to July 7, 2009, and the embryo: 1) was created using in vitro fertilization for reproductive purposes and was no longer needed for this purpose; and 2) was donated by individuals who sought reproductive treatment ("donor(s)") who gave voluntary written consent for the human embryo to be used for research purposes. The applicant is advised that the Working Group of the Advisory Committee to the NIH Director will consider submitted materials taking into account the principles articulated in Section II(A) of the NIH Guidelines for Human Embryonic Stem Cell Research, 45 CFR 46 Subpart A, and the following points to consider: during the informed consent process, including written and oral communications, whether the donor(s) were: (1) informed of other available options pertaining to the use of the embryo; (2) offered any inducements for the donation of the embryo; and (3) informed about what would happen to the embryo after the donation for research.

Please confirm that you have received this request and let me know if you have any questions.

Sincerely,

Ellen Gadbois

Ellen T. Gadbois, Ph.D.
Office of Science Policy Analysis
Bldg 1 Room 218D
National Institutes of Health
voice: 301-594-2567
fax: 301-402-0280

From: HESCREGISTRY (NIH/NICHD)
Sent: Friday, February 19, 2010 11:31 AM
To: wcaldwel@advancedcell.com; ksingh@advancedcell.com
Subject: New hESC Registry Application Request #2010-ADM-006

To: William Caldwell (Signing Official)
From: Kathy Singh
To: HESCREGISTRY (NIH/NIDCD)
Cc: William Caldwell
Subject: RE: New hESC Registry Application Request #2010-ADM-006
Date: Wednesday, May 26, 2010 5:04:06 PM

Dear Dr. Gadbois,

I am responding to your question below. The embryos were transported to us on April 10, 2007.

I will await your submission information later.

Sincerely,
Kathy Singh

From: HESCREGISTRY (NIH/NIDCD) [mailto:hescregistry@mail.nih.gov]
Sent: Wednesday, May 26, 2010 11:56 AM
To: Kathy Singh
Cc: HESCREGISTRY (NIH/NIDCD); William Caldwell
Subject: RE: New hESC Registry Application Request #2010-ADM-006

Hello Ms. Singh,

Thank you very much for your response and the blank consent form. (For the other submissions, if the redaction removes text beyond the signatures, It would be helpful to have a blank form included as you did here.)

The Working Group has one further question: can you provide the date on which the embryos were transferred from the IVF clinic to ACT? The Working Group would like to know how long the donors actually had to change their minds about the embryo donation. (We know that the research consent form was signed on 1/19/07, and the consent form states that the average time period between signing the consent form and transferring the embryos to ACT is two weeks, but the Working Group would like to know that actual time period in this instance.)

Thank you again for your assistance. We are hoping to have this submission ready for consideration by the full Advisory Committee to the Director at their June 10 meeting. That means the ACD Working Group will need to have this information very soon in order to finalize their report to the ACD.

I also will be sending you more information later today about preparing your submission for the June 10 meeting, and information about the meeting itself, which is public and can be viewed by webcast.

Sincerely,
Ellen Gadbois

Ellen L. Gadbois, Ph.D.
Office of Science Policy Analysis
Bldg 1 Room 218D
National Institutes of Health
Voice: 301-594-2667
Fax: 301-402-0280

From: Kathy Singh [mailto:KSingh@advancedcell.com]
Sent: Tuesday, May 25, 2010 6:13 PM
To: HESCREGISTRY (NIH/NIDCD)
Subject: RE: New hESC Registry Application Request #2010-ADM-006

Dear Dr. Gadbois,

I have been able to obtain answers to the questions you asked. Please see my responses in black below. Thank you again for your review.

Best,
Kathy Singh