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

NOTE: Duplicative information in the submission is not included.

hESC Registry Application Database

Detailed Listing for Request #: 2010-ACD-007
May 18, 2010

hESC Registry Application Search Results

Request #: 2010-ACD-007
Status: Pending

Review: ACD

Assurance: Yes (Section II(B))
Certification: Yes
Authority: Yes

Cell Lines: 1
Available: 1

Previous #:
2010-DRAFT-005
2010-ADM-006

Email
Edit
Delete
Switch to ADM

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 / ksingh@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: ksingh@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 6: (PDF - 05/11/2010) Consent for IVF
Document 7: (PDF - 05/11/2010) Derivation Protocol
Document 8: (PDF - 05/11/2010) Blastocyst Grading for Transfer
Document 9: (PDF - 05/11/2010) Consent for Cryopreservation
Document 10: (PDF - 05/11/2010) Transfer of Frozen Embryos
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_Applicaton_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\williamcaldwell **On:** 02/19/2010 | **Last Updated By:** NIH\hannemannd **On:** 05/17/2010 | **Record ID:** 51

Total Record Count = 1

Administration Page

Logout of NIH Form 2890 Admin Site

ADVANCED CELL TECHNOLOGY
P.O. Box 1700 Santa Monica, CA 90406 Ph 310-576-0611 Fax 576-0662

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

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Revised 7/24/06

ACT IRB #: 901
APPROVED ON: JUL 27 2006
VALID THROUGH: MAR 28 2007

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).

381 Plantation Street, Biotech V, Worcester, MA 01605 Tel 508.756.1212 Fax 508.756.4468

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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.

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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.

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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.

ACT IRB # 001 APPROVED ON: JUL 27 2006 VALID THROUGH: MAR 28 2007

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.

Over-view of In Vitro Fertilization ( )
IVF Hurdles
Multiple Gestation and Multifetal Pregnancy Reduction ( )
Side effects of Gonadotropins ( )
Intracytoplasmic Sperm Injection (ICSI)
Assisted Hatching ( )
Blastocyst 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 risks 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 also give 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) 12-14-05 (Date)

(Partner-print) 12-14-05 (Date)

(Witness-print) (signature) 12/14/05 (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 [redacted] to ensure the safety of frozen embryos. 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 or children is/are born, there exists the possibility that he/she/they may in some way be abnormal. We release the [redacted] and personnel 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 both of us, we have chosen to:

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

Patient Printed Name [redacted] Patient Signature [redacted] 12/14/05 Date ID – specify

Witness Printed Name/Title [redacted] Witness Signature [redacted] 12/14/05 Date

Patient Printed Name [redacted] [redacted] 12/14/05 Date ID – specify

Witness Printed Name/Title [redacted] Witness Signature [redacted] 12/14/05 Date

If signing this form outside of [redacted] I must be notarized

I certify that I know or have satisfactory evidence that: _____

Is/are the person (s) who appeared before me, and said person (s) acknowledged that he/she/they signed this instrument And acknowledged it to be his/her/their free and voluntary 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: _____

PATIENT: _____ Patient No.: _____

law guarantees that you have the *right* and *obligation* to make decisions concerning your health care. Your physician can provide you with the necessary information and advice, but as a member of the health care team, you must enter into the decision making process. This form has been designed to acknowledge your acceptance of treatment recommended by your physician.

1. I hereby authorize Dr. _____ and/or such associates, assistants or designees, including medical residents in training as may be selected by said physician to treat the following condition(s) which has (have) been explained to me: *(Explain the nature of the condition(s) in professional and lay language.)*

infertility

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.)*

transvaginal oocyte retrieval

3. I recognize that, during the course of the operation, post operative care, medical treatment, anesthesia or other procedure, unforeseen conditions may necessitate additional or different procedures than those above set forth. I therefore authorize my above named physician, and his or her assistants or designees, to perform such surgical or other procedures as are in the exercise of his, her or their professional judgment necessary and desirable. The authority granted under this paragraph shall extend to the treatment of all conditions that require treatment and are not known to my physician 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 severe loss of blood, infection and cardiac arrest that can lead to death or permanent or partial disability, which may be attendant to the performance of any procedure. I acknowledge that no warranty or guarantee has been made to me as to result or cure.

5. FULL DISCLOSURE
I certify that my physician has informed me of the nature and character of the medical procedure or surgery described on this form, including its possible significant risks, complications and anticipated results; and the alternative forms of treatment, including non-treatment and their significant risks, complications and anticipated results.

6. LIMITED DISCLOSURE-to be signed by patient if patient elects not to be informed.
I certify that my physician has explained to me and I have the right to have clearly described to me the nature and character of the proposed medical procedure or surgery described on this form, including its possible significant risks, complications and anticipated results; and the alternative forms of treatment, including non-treatment, and their significant risks, complications and anticipated results.
I do not wish to have these risks and facts explained to me.

_____
PATIENT/OTHER LEGALLY RESPONSIBLE PERSON SIGN IF APPLICABLE

7. I consent to the administration of anesthesia by my attending physician, by an anesthesiologist, or other qualified party under the direction of a physician as may be deemed necessary. I have been informed by my physician and understand that all anesthetics involve risks of complications and serious possible damage to vital organs such as the brain, heart, lung, liver and kidney and that in some cases may result in paralysis, cardiac arrest and/or brain death from both known and unknown causes.

8. I consent to the transfusion of blood and blood products as deemed necessary. I understand that all blood and blood products involve risks of a reaction, bruising, fever, hives, and in rare circumstances infectious diseases such as hepatitis and HIV/AIDS. I understand that precautions are taken by the _____ in screening donors and in matching blood for transfusion to minimize risks.

9. Any tissues or parts surgically removed may be disposed of by the hospital or physician in accordance with accustomed practice. Any biological specimens, such as tissue, blood, bodily fluids, etc. may be disposed of or used for medical study, medical procedure or in research.

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 by me to the patient or his/her representatives before the patient or his/her representatives consented.

PHYSICIAN'S SIGNATURE _____ DATE 12/14/05 TIME 1000

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 explanations referred to were made and all blanks or statements requiring insertion or completion were filled in before I affixed my signature.

SIGNATURE OF PATIENT OR PATIENT REPRESENTATIVE _____ DATE 12/12/05 TIME 0940

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 0940

NAME PLATE

SPECIAL INSTRUCTIONS FOR OPERATIVE, POST-OPERATIVE, PAIN, MEDICAL TREATMENT, ANESTHESIA OR OTHER INVASIVE PROCEDURES:

38

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 BS 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 incubators 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 4×, 10×, 20×, and HMC 20× and 40×. HMC is recommended for viewing ES cells and is required for embryo evaluation.

Heated microscope stage for both stereo and inverted microscope: A (Nikon) slide warmer keeps extra PMEF 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 (CO_2 concentration, humidity, and temperature) need to be checked daily with external monitoring equipment.

External monitoring equipment:

- Surface thermometer
- Mercury liquid-immersed thermometer

Essentials of Stem Cell Biology

- Hygrometer
- CO_2 monitor and gas calibration kit (GD 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 CO_2 (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 90% 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 Steris, Mentor, Ohio).

If it comes to the worst, the triple-action drug Normocin (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 Plasmanate, 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 osmolarity higher than 470 mOsm/kg and endotoxicity lower than 0.9 EU/ml were the best. Upon thawing, make single-use aliquots and freeze.
- Plasmanate (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 (1000×) solution (Invitrogen, Cat. No. 21985-023)
- Non-essential amino acids (NEAA), 100x solution (Invitrogen, Cat. No. 11140050)
- Penicillin–streptomycin, 100× solution (Invitrogen, Cat. No. 15070-063)
- Glutamax-I, 100× solution, a stable dipeptide of L-glutamine and L-alanyl, 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 ml 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 ng/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–0.53 mM EDTA (Invitrogen, Cat. No. 25300-054)
- Gelatin from porcine skin (Sigma, Cat. No. G1880)
- Phosphate-buffered saline (PBS), Ca2+, Mg2+-free (Invitrogen, Cat. No. 14190-144)
- Normocin, an antibiotic active against gram +/– bacteria that also has antimycoplasma and antifungi activity (Invivogen, San Diego, California; Cat. No. ant-nr-2; comes as a 500× solution)

MEDIA RECIPES

Bottles of media that are opened frequently become alkali 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

hES Cell Derivation Medium

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

o 100 ml of basal medium, add:

ml Plasmanate
ml Serum Replacement
ml FBS
0 µl of human LIF (final concentration 20 ng/ml)
20 µl of bFGF stock solution (final concentration 8 ng/ml), r more (up to 20 ng/ml)
terilize by 0.22-µm filtration

hES Cell Growth Medium

200 ml of basal medium, add:

0-ml Plasmanate
0-ml Serum Replacement
240 µl of human LIF for 10 ng/ml, or 480 µl for 20 ng/ml
120 µl of bFGF stock solution (final concentration 4 ng/ml) for 1× bFGF, or more if a higher concentration is desired
Sterilize by 0.22-µm filtration

Gelatin

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

Mitomycin C

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

SCREENING MEDIA COMPONENTS

t is important to be consistent with screening, aliquoting, and torage of the media components. Various lots of Serum Replacement, Plasmanate, 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, Plasmanate, 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 grow 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 refractory borders. The colonies appear deep red when stained for alkaline phosphatase activity. In differentiating colonies, the cells are more loosely packed, with diffuse 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 are 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 postcoitus (dpc) (ICR) mouse embryos as described in Robertson (1987). The 12.5 dpc embryos are eviscerated, 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 decreases as they go through multiple passages; therefore, thawed PMEFs are only passaged 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 plated on gelatinized plates in PMEF growth medium. In

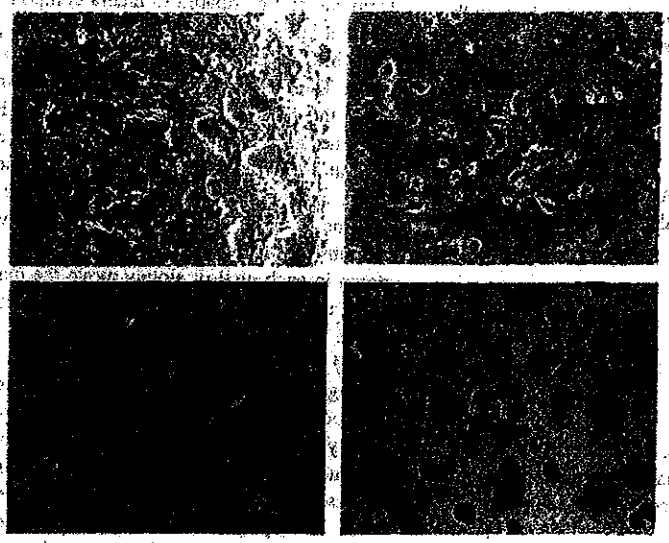

Figure 38-1. Media testing. (A and C) Comparison of 16% serum replacement with (B and D) 8% Serum Replacement and 8% plasmanate. The quality of the media supplements is assessed by (A and B) evaluating the morphology of the colonies under phase contrast, as described in the text, followed by (C and D) staining for alkaline phosphatase activity. Note that although the morphology of the colonies in panels A and B is comparable, the activity of alkaline phosphatase is higher in (D) the medium with both Serum Replacement and plasmanate. Magnification 40x.

serum-free hES cell growth media, the PMEFs 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 50,000 to 60,000 cells/cm^2. We prefer to use plates of PMEFs 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 dispase in conjunction with mechanical dispersion. Mechanical dispersion can provide colonies of "perfect" morphology, as it permits one 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, thus 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 trypsin. 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. 15601 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 38-2 shows examples of mechanically passaged cultures.

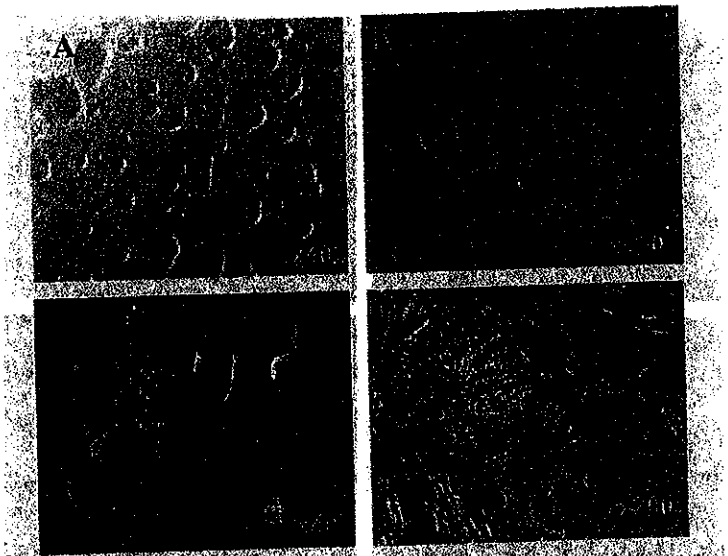

Figure 38-2. Morphology of mechanically dispersed hES cells. Colonies in panels A and B show no signs of differentiation. Note that the cells are small and tightly packed and that the colonies have sharp borders. In panel C, the long arrow points to a much differentiated colony that should not be dispersed; short arrows show partially differentiated colonies that can be dispersed and may produce undifferentiated colonies at the next passage. Panel D shows higher magnification of the framed colonies in panel C, shifted 90 degrees. The arrow points to a partially differentiated colony, which has become multilayered in the middle. The other colony is undifferentiated and is similar to the colonies shown in panels A and B.

pointing out parts of colonies that have differentiated and should be avoided when passaging the culture.)

Derivation of hES Cells

Many factors that influence whether an isolated ICM will produce an hES cell line are not fully understood. Some of the factors to consider are what stage the embryo was frozen in 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 must be determined empirically, usually occurring between day 5 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 sucking 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): Aliquoted and stored at –80°C; freshly diluted 1:10 in hES cell derivation medium
- Complement (Sigma, Cat. No. S1639): Aliquoted and stored at –80°C; freshly diluted 1:10 in derivation medium
- Capillaries for embryo transfer: Thinly drawn capillaries (approximately the diameter of the ICM) for the trophectoderm removal

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 CO_2 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

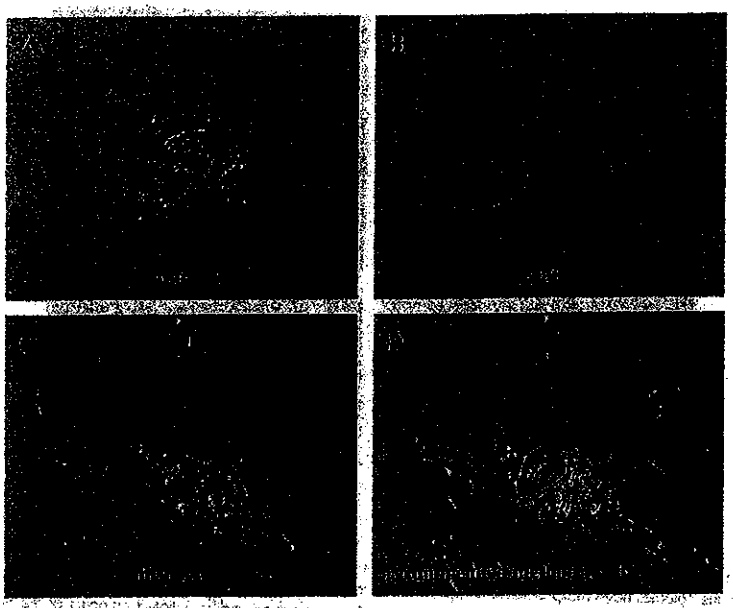

Figure 38-3. Initial ICM outgrowth. The initial outgrowth of the ICM rarely shows typical ES colony morphology and often includes many differentiated-looking cells. When no obvious ES cell-like colonies large enough for dispersion can be located, more time is required before the first colony dispersion can be done (A and B) The initial ICM outgrowth of two future hES lines. At this stage, when the dispersion is attempted, the outgrowth and the PMEF monolayer come up together, so it is better to wait before dispersing. (C) The ICM is ready for dispersion when a colony of ES-like cells is large enough to be dispersed into several pieces, leaving 20 to 50% of the outgrowth on the original place for future regrowth. (D) Lines transcribe the number of pieces recommended for the dispersion of this colony. A narrow capillary is used, and a small part of the colony is left untouched (arrow). Magnification 100x.

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 in the CO_2 incubator 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 derivation 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 thinly drawn capillary (about the diameter of the ICM); the lysed trophectoderm 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 immunosurgery was done because the trophectoderm was not sufficiently intact.

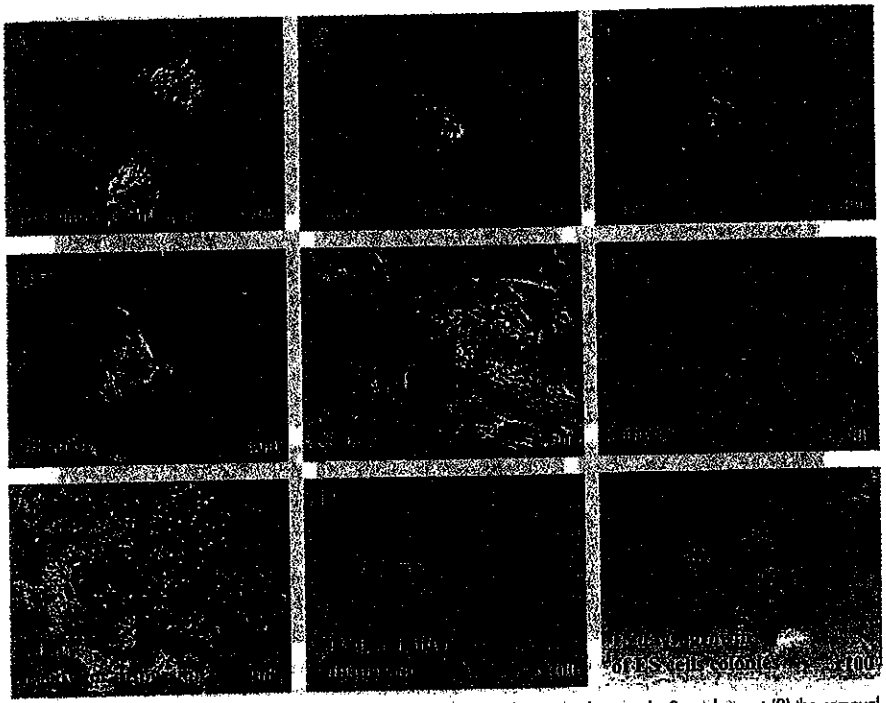

Figure 38-4. Early stages of hES cells derivation. (arrow in panel A) A blastocyst of poor quality, grade 3, underwent (B) the removal of the zona pellucida. (C) The next day, after being plated on the PMEF monolayer, it has attached. (D) Forty-eight hours after plating, the ICM appears smaller, possibly because of some cell death. (E) Four days after plating, an outgrowing group of cells is visible with some small cells in the middle (arrow), (F) which become less visible two days later. (G) Ten days after plating, a small colony of ES-like cells (arrow) has formed within the large group of differentiated-looking cells and is now large enough for dispersion. (H) Original ICM outgrowth two days after it was dispersed; note regrowth of small ES-like cells in the cleared area (arrow), which are ready for another dispersion. (I) Formation of ES cell colonies from the recombined first and second dispersion.

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 PMEF 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 cells 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 pluripotential 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.

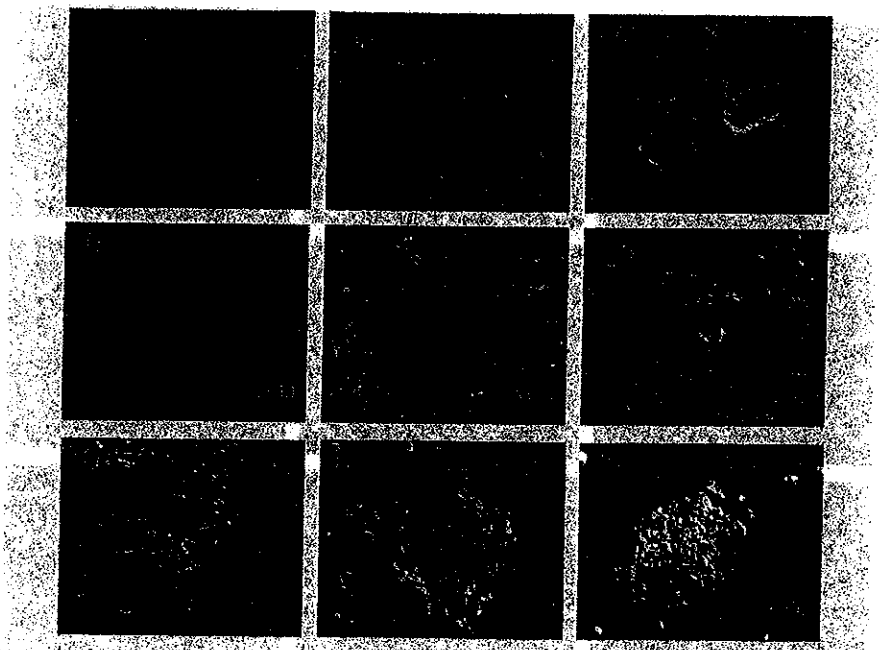

Figure 38-5. Approaches for dispersion of colonies with different morphologies. Various morphologies of hES cell colonies encountered at early stages of derivation when mechanical dispersion can be the tool of choice used to selectively pick undifferentiated colony parts. (A) All colonies are undifferentiated 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 (the centers 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 badly 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. Adaptation of mechanically passaged hES cells to trypsin. (A) After five days of growth, colonies were mechanically dispersed and transferred to a fresh plate of PMEFs. (B) Morphologically, they are similar to the original plate. (C) The remaining colonies in 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

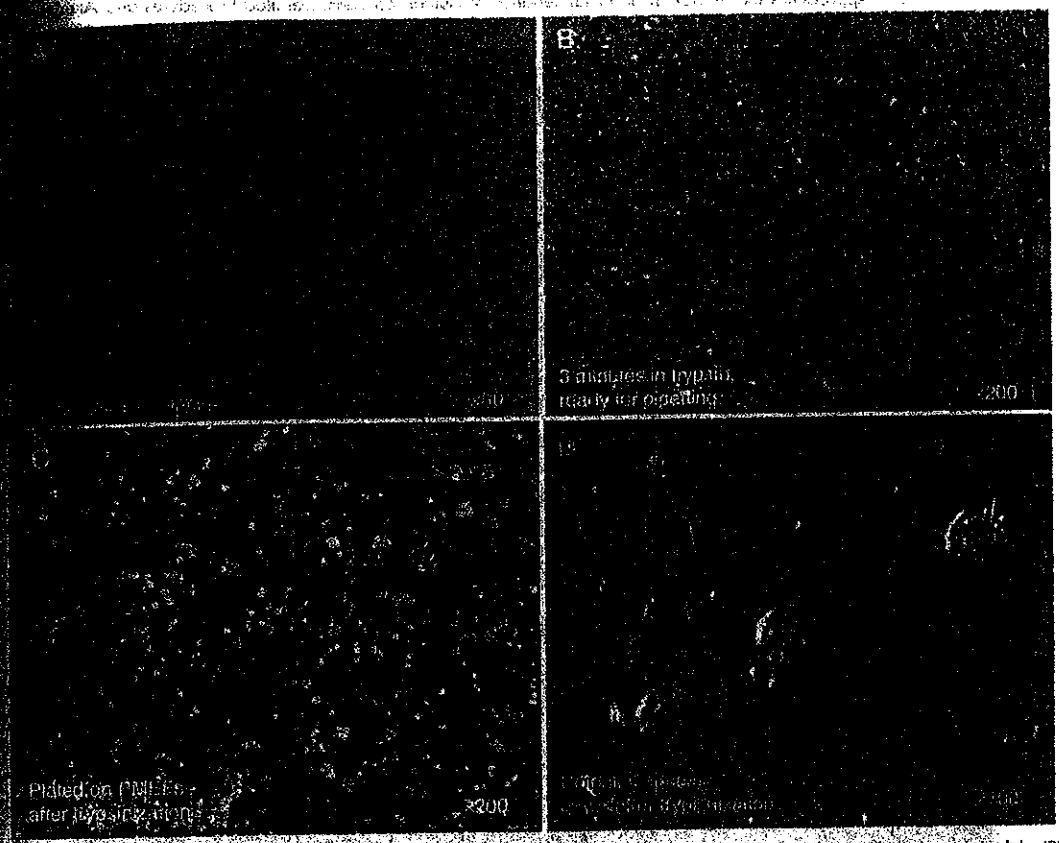

Figure 38-7. Passaging by trypsinization. (A) After 1.5 minutes in trypsin, the PMEFs look shrunken, and the hES colonies have loosened a little. (B) After 3 minutes in trypsin at higher magnification, the colonies are less compact; this is a good time to begin pipetting. Please note that depending on the density of the colonies, the days after passaging, and the degree of differentiation, the time required to reach this stage may vary and needs to be worked out empirically. (C) Suspension of hES cells after replating onto fresh PMEFs. Note that most cells are in small cell aggregates. (D) Small colonies begin to appear on day 2 after plating (at a 1:3 ratio). The time at which the first colonies are seen may vary depending on the splitting ratio and the size of the cell aggregates.

clumps 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 pipetteman (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

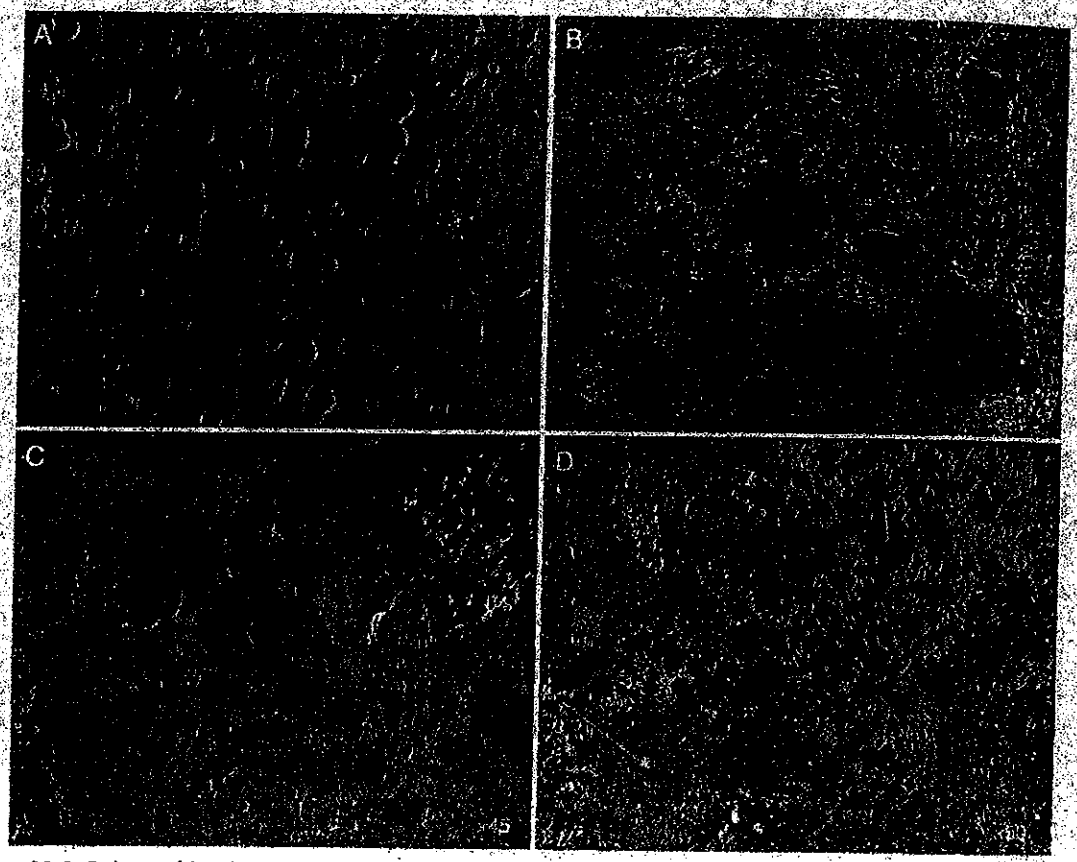

Figure 38-8. Evaluation of the culture prior to trypsinization. (A and B) Mostly undifferentiated hES cells are ready for trypsinization. (C) The colonies are a little overgrown and show signs of differentiation but still can be safely passaged with trypsin. (D) Badly differentiated hES cells (an arrow points to a colony) can still be rescued by mechanically picking colony pieces and passaging.

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 160 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.

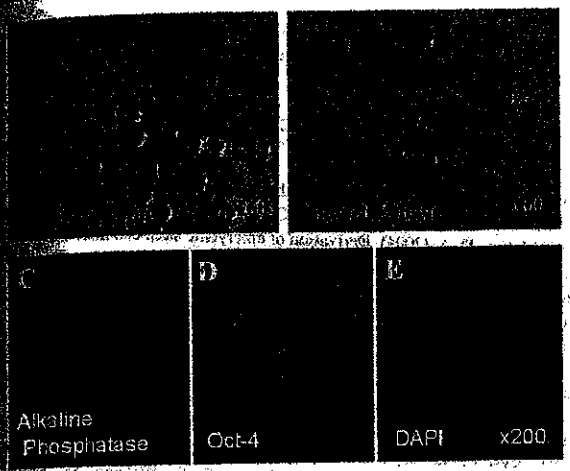

Figure 38-9. Freezing and thawing of hES cells. (A) The approximate density and morphology of the colonies of hES cells at freezing. (B) When thawed onto the same diameter plate, the colonies should be ready for the next split in 5–7 days. (C and D) Thawed hES cells show high expression of *Oct-4* and alkaline phosphatase. (E) same field as C and D, DAPI-stained

FREEZING PROTOCOL

Select a high-quality confluent culture with good morphology for freezing. We also recommend taking a picture of a sample field and staining for molecular markers characteristic of undifferentiated hES cells for future reference.

Materials needed:

- Chilled freezing medium: 90% FBS, 10% DMSO
- Cryovials, labeled with the line, passage number, and date
- Cryovial rack (rack with ice reservoir by Corning)
- Styrofoam rack from packaging for 15-ml centrifuge tubes
- –80°C freezer

1. Trypsinize the cells; centrifuge in PMEF medium (see earlier explanation).
2. Resuspend the pellet in the cold freezing medium. We recommend freezing one confluent 35-mm plate per vial in 0.5 ml of freezing medium. Work quickly and keep the cells on ice after the addition of the freezing medium.
3. Aliquot cell suspension into prechilled freezing vials and sandwich the vials between two Styrofoam racks; tape to prevent the two racks from separating and transfer to a –80°C freezer overnight. Transfer the cryovials to liquid nitrogen for long-term storage.

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.
2. Make a thawing medium. We use 70% hES cell growth medium supplemented with 2× 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 CO_2 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 pipetteman, 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 pipetteman (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.

Immunosurgery A method of removing the trophectoderm of a blastocyst using antibodies bound to the surface antigens of the trophectoderm and complement.

PMEF (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 Witmyer, for contributing their invaluable experience 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

Cowan, C. A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J. P., Wang, S., Morton, C. C., McMahon, A. P., Powers, D., and Melton, D. A. (2004). Derivation of embryonic stem cell lines from human blastocysts. *New Engl. J. Med.* 350, 1353–1356.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat. Biotechnol.* 18, 399–404. [Erratum in *Nat. Biotechnol* 18(5), 559].

Robertson, E. J. (ed.) (1987). *Teratocarcinomas and embryonic stem cells: a practical approach*. IRL Press: Oxford.

Solter, D., and Knowles, B. B. (1975). Immunosurgery of mouse blastocyst. *PNAS* 72, 5099–5102.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. [Erratum in *Science* (1998). 282(5395), 1827].

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 trophectoderm using the following system.

Degree of Expansion

EB1: The blastocoel filling less than half of the volume of the embryo
EB2: The blastocoel filling greater that half of the volume of the embryo
3: The blastocoel 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

Trophectoderm 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.
5. 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.
8. Close shipping container and call Fed-Ex for pickup. Always ship priority overnight.

From: Hannemann, Diane (NIH/OD) [C]
To: Hannemann, Diane (NIH/OD) [C]
Subject: FW: New hESC Registry Application Request #2010-ADM-006
Date: Tuesday, May 11, 2010 10:15:53 AM
Attachments: Blastocyst Grading for Transfer.pdf
Consent for Cryopreservation.pdf
Consent for IVF.pdf
Transfer of Frozen Embryos.pdf
Derivation Protocol.pdf

From: Kathy Singh [mailto:KSingh@advancedcell.com]
Sent: Monday, May 10, 2010 11:09 AM
To: Gadbois, 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. Gadbois,
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 it 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, "Blastocyst Grading for Transfer" and "Transfer of Frozen Embryos".

☆ Additional information from ACT

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 12/2/05; IVF clinic froze the embryos on 12/20/05; embryos were donated on 1/19/07.

☆

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 2006. 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 Pluripotentent 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 L. 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: wcaldwell@advancedcell.com; ksingh@advancedcell.com
Cc: HESCREGISTRY (NIH/NIDCD)
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 sending 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 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) 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 L. 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/NIDCD)
Sent: Friday, February 19, 2010 11:31 AM
To: wcaldwell@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 19, 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 transfering 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: 301-594-2567
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