Materials Submitted to NIH
from Cellartis
Submission #2010-ACD-010

NOTE: Duplicative information in the submission is not included. Original documents in Sweden with signatures also not included.

(1)

hESC Registry Application Database

Detailed Listing for Request #: 2010-ACD-010
November 30, 2010

hESC Registry Application Search Results

Request #: 2010-ACD-010
Status: Pending

Review: ACD

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

Cell Lines: 3
Available: 3

Previous #:
2009-DRAFT-025
2010-ADM-005

Email
Edit
Delete
Switch to ADM

Organization: Cellartis AB
Org Address: Arvid Wallgrens backe 20 413 46 Göteborg Sweden
DUNS: 507705726 **Grant Number(s):**
Signing Official (SO): Johan Hyllner / +46-31-7580902 / johan.hyllner@cellartis.com
Submitter of Request: Katarina Emanuelsson / +46-31-7580931 / katarina.emanuelsson@cellartis.com
Submitter Comments: (None)

Line #1: SA001
NIH Approval #:
Available: Yes
Embryo from U.S.: No
Embryo Donated in Year(s): 2001
Provider Name: Cellartis AB
Provider Phone: +46-31-758 09 00
Provider Email: info@cellartis.com
Provider URL: http://www.cellartis.com/
Provider Restrictions: No clinical or commercial use.

NIH Restrictions:

Additional Information:

Line #2: SA002
NIH Approval #:
Available: Yes
Embryo from U.S.: No
Embryo Donated in Year(s): 2001
Provider Name: Cellartis AB
Provider Phone: +46-31-758 09 00
Provider Email: info@cellartis.com
Provider URL: http://www.cellartis.com/
Provider Restrictions: No clinical or commercial use.

NIH Restrictions:

Additional Information: The karyotype for SA002 is 47XX (+13).

Line #3: SA002.5
NIH Approval #:
Available: Yes
Embryo from U.S.: No
Embryo Donated in Year(s): 2001
Provider Name: Cellartis AB
Provider Phone: +46-31-758 09 00
Provider Email: info@cellartis.com
Provider URL: http://www.cellartis.com/

(2)

Provider Restrictions: No clinical or commercial use.

NIH Restrictions:

Additional Information:

Supporting Documents:
Document 1: (PDF - 02/19/2010) Summary of Supporting Information - Elements: 16
Document 2: (PDF - 02/19/2010) IRB Application and Approval - Elements: 16
Document 3: (PDF - 02/19/2010) Donor consents - Elements: 1,2,3,5,8,9,10,11,13
Document 4: (PDF - 02/19/2010) Certification of signed informed consent - Elements: 7,16
Document 5: (PDF - 02/19/2010) Assurance of Conditions SA001, SA002, SA002.5 - Elements: 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15
Document 6: (PDF - 02/19/2010) Making SA001 and SA002 fully anonymous (un-tracable) - Elements: 6,10,14,15,16
Document 7: (PDF - 02/19/2010) Cell Line Description SA001 - Elements: 7,16
Document 8: (PDF - 02/19/2010) Cell Line Description SA002 and SA002.5 - Elements: 7,16
Document 9: (PDF - 02/19/2010) Derivation, Characterization and Differentiation of hESC - Elements: 16
Document 10: (PDF - 02/19/2010) Sample Letter of SO - Elements: 16

Administrative Comments: SO certifications updated by E. Gadbois 26 Feb 2010

Email correspondence 30 Jun 2010 uploaded by D. Hannemann 30 Jun 2010

Procedure uploaded by D. Hannemann 30 Jun 2010

Informed Consent uploaded by D. Hannemann 30 Jun 2010

Certification of Signed Consent uploaded by D. Hannemann 30 Jun 2010

Assurance of Conditions uploaded by D. Hannemann 30 Jun 2010

Switched to ACD review per Dr. Landis by E. Gadbois 8 July 2010

NIH Staff IIB Analysis uploaded by D. Hannemann 12 Jul 2010

Submitter Response email 13 Jul 2010 uploaded by D. Hannemann 13 Jul 2010

IIB Assurance uploaded by D. Hannemann 13 Jul 2010

27 July 2010 Submitter Response email uploaded by D. Hannemann 27 July 2010

19 August 2010 Submitter Response email uploaded by D. Hannemann 20

Aug 2010

19 Aug 2010 email attachment - Donor Consent (English translation) uploaded by D. Hannemann 20 Aug 2010

19 Aug 2010 email attachment - Donor Consent (Orig Swedish) uploaded by D. Hannemann 20 Aug 2010

19 Aug 2010 email attachment - Assurance of Conditions uploaded by D. Hannemann 20 Aug 2010

24 Aug 2010 Email from submitter uploaded by D. Hannemann 17 Sept 2010

Administrative Attachments:
Document 1: (PDF - 06/30/2010) Email Correspondence 30 Jun 2010
Document 2: (PDF - 06/30/2010) Procedure for Establishment of Lines
Document 3: (PDF - 06/30/2010) Informed Consent Form
Document 4: (PDF - 06/30/2010) Certification of Signed Consent
Document 5: (PDF - 06/30/2010) Assurance of Conditions
Document 6: (PDF - 07/08/2010) Record of decision to switch to ACD
Document 7: (DOC - 07/12/2010) NIH Staff Summary
Document 8: (PDF - 07/13/2010) Submitter Response Email 13 Jul 2010
Document 9: (PDF - 07/13/2010) IIB Assurance
Document 10: (PDF - 07/27/2010) 27 July 2010 Submitter Response Email
Document 11: (PDF - 08/20/2010) 19 Aug Submitter Response email
Document 12: (PDF - 08/20/2010) Donor Consent (English) - 19 Aug email attacment
Document 13: (PDF - 08/20/2010) Donor Consent (Swedish) - 19 Aug email attachment
Document 14: (PDF - 08/20/2010) Assurance of Conditions - 19 Aug email attachment
Document 15: (PDF - 09/17/2010) 24 Aug 2010 email from submitter

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Status History:
Draft: 10/21/2009
Pending: 02/19/2010

Emails Sent: 02/19/2010-New_Applicaton_Email

Previous ADM Request Number: 2010-ADM-005
Switched from ADM to ACD Date: 07/08/2010
Reason for Switch to ACD Review:
There are several elements of Section IIA that the administrative review group believes are not clearly met by the documentation provided and unlikely to be satisfied by further information from Cellartis: - Element 11: During the consent process, the donor(s) were informed of the following:... hESCs derived from the embryos might be kept for many years. - Element 14: During the consent process, the donor(s) were informed of the following... The results of research using the hESCs may have commercial potential, and that the donor(s) would not receive financial or any other benefits from any such commercial development.

There are other additional elements that are currently not met, but might be met depending on further information from the submitter. There is also

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	an added complexity that the donor couples were consented a total of four times.
	Added By: Commons\HYLLNER **On:** 10/21/2009 | **Last Updated By:** NIH\hannemannd **On:** 11/30/2010 | **Record ID:** 25

Total Record Count = 1 *

Administration Page

Logout of NIH Form 2890 Admin Site

cellartis

Signature of the Signing Official for Certification and Assurance Required for hESC Registry Request -NIH Form 2890.

Updated: 07/01/2010

Date: 13th of July, 2010

NIH Stem Cell Registry:

I hereby certify that the statements in the Request for Human Embryonic Stem Cell Line to be Approved for Use in NIH Funded Research (NIH Form 2890), submitted by Cellartis (Catharina Ellerström), and below, are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties (U.S. Code, Title 18, Section1001).

I further confirm that that I have the authority and/or rights pertaining to the human embryonic stem cell line(s) identified in item 6 of the form to make this request for NIH review and determination of eligibility for use in NIH funded research (e.g., I am the owner, deriver or licensee or have written permission of the same to submit). Any and all restrictions on the use of the stem cell line are clearly and completely identified in item 8 of the form.

Assurance Statements (mark the appropriate statement with an "X"; you may only check **one** Assurance statement.):

_____ **Assurance in accord with Section II(A) of the NIH Guidelines:**

I hereby assure that the donation of the embryo from which the cell line(s) identified in item 6 was derived was in accordance with the elements of Section II(A) of the NIH Guidelines on Human Stem Cell Research.

OR

__X__ **Assurance in accord with Section II(B) of the NIH Guidelines:**

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[1].

OR

_____ **Assurance in accord with Section II(C) of the NIH Guidelines:**

[1] 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 during research.

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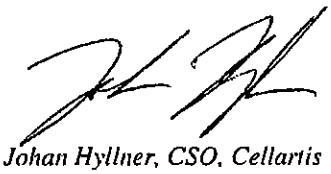
(6)

cellartis

I hereby assure that the embryo from which the cell line(s) identified in item 6 of the form was derived was donated outside the United States on or after July 7, 2009, and the alternative procedural standards of the foreign country where the embryo was donated provide protections at least equivalent to those provided by Section II(A) of the NIH Guidelines on Human Stem Cell Research.

I acknowledge that I have read, understood, and agreed to the information provided on the form, including the Instructions for completing the form, and the Certification, Authority and Assurance provided above.



Johan Hyllner, CSO, Cellartis

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Assurance of Conditions of Consent for human Embryonic Stem Cell lines SA001, SA002 and SA002.5 (Subclone of SA002)

As responsible for the stem cell program at Sahlgrenska University Hospital and for ensuring that the consent from the embryo donors whose embryos resulted in the derivation of the SA001, SA002 and SA002.5 (subclone of SA002 accordingly having the donor consent as SA002) human Embryonic Stem Cell (hESC) lines were obtained, I, Charles Hanson, Associate Professor Sahlgrenska University Hospital along with Lars Nilsson, Associate Professor attending physician at Sahlgrenska University Hospital at the time of donation, hereby provide the following written assurance that the embryos used for the derivation of the SA001 and SA002:

1. were created using in vitro fertilization for reproductive purposes and were no longer needed for this purpose;

2. were donated by individuals who sought reproductive treatment (hereafter referred to as "donor(s)") and who gave voluntary written consent for the human embryos to be used for research purposes.

Furthermore, during the consent process:

a. All options available in the health care facility where treatment was sought pertaining to the embryos no longer needed for reproductive purposes were explained to the individual(s) who sought reproductive treatment.

b. No payments, cash or in kind, were offered for the donated embryos.

c. Policies and/or procedures were in place at the health care facility where the embryos were donated that neither consenting nor refusing to donate embryos for research would affect the quality of care provided to potential donor(s).

d. There was a clear separation between the prospective donor(s)'s decision to create human embryos for reproductive purposes and the prospective donor(s)'s decision to donate human embryos for research purposes. Specifically:

    i. Decisions related to the creation of human embryos for reproductive purposes should have been made free from the influence of researchers proposing to

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derive or utilize hESCs in research. The attending physician responsible for reproductive clinical care and the researcher deriving and/or proposing to utilize hESCs should not have been the same person unless separation was not practicable.

ii. At the time of donation, consent for that donation should have been obtained from the individual(s) who had sought reproductive treatment. That is, even if potential donor(s) had given prior indication of their intent to donate to research any embryos that remained after reproductive treatment, consent for the donation for research purposes should have been given at the time of the donation.

iii. Donor(s) should have been informed that they retained the right to withdraw consent for the donation of the embryo until the embryos were actually used to derive embryonic stem cells or until information which could link the identity of the donor(s) with the embryo was no longer retained, if applicable.

e. During the consent process, the donor(s) were informed of the following:
   i. that the embryos would be used to derive hESCs for research;
   ii. what would happen to the embryos in the derivation of hESCs for research;
   iii. that hESCs derived from the embryos might be kept for many years;
   iv. that the donation was to be carried out according to Swedish law on Discrimination SFS 2008:567 (This law replaces the Swedish law on equality (SFS 1991:433) and six other civil rights laws in force at the time of establishment.) Those laws do not allow discrimination, any restriction or direction regarding the individual(s) who may receive medical benefit from the use of the hESCs now or at the time of establishment. Additionally, referring to clinical matters such as who may be the recipients of cell transplants, hESC lines SA001 and SA002 where derived for research purposes only and are not for clinical use;
   v. that the research was not intended to provide direct medical benefit to the donor(s);
   vi. that the results of research using the hESCs would be carried out under Swedish law (at that time the Swedish law SFS 1991:115 , now replaced by the law on Genetic Integrity SFS 2006:351. This law allows commercialization of non-traceable (anonymous) human embryonic stem cell lines), and that the donor(s) would not receive financial or any other benefits from any such commercial development;
   vii. that the identity of the donor(s) would not be provided to researchers.

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To the best of my knowledge, I, Charles Hanson, Associate Professor Sahlgrenska University Hospital along with Lars Nilsson , Associate Professor Sahlgrenska, attending physician at Sahlgrenska University Hospital at the time of donation, hereby certify that the above represents a true, accurate and complete description of the consent process used for the donation of the embryos which resulted in the derivation of the SA001 and SA002 human Embryonic Stem Cell lines. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties (U.S. Code, Title 18, Section 1001).

Charles Hanson, Associate Professor Date 100129

Reproductive Medicine
Department of Obstretrics and Gynaecology
Institution of Clinical Sciences, Sahlgerenska Academy
Sahlgrenska University Hospital
S-413 45 GÖTEBORG
SWEDEN

Lars Nilsson, MD, Associate Professor Date 100129

Reproductive Medicine
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Institution of Clinical Sciences, Sahlgerenska Academy
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SWEDEN

(10)

Summary of Supporting Information Provided for human Embryonic Stem Cell (hESC) Lines SA001, SA002 and SA002.5 (subclone of SA002) from Cellartis AB

Document 1: ***Summary of Supporting Information Provided for human Embryonic Stem Cell Lines SA001, SA002 and SA002.5 (subclone of SA002) from Cellartis AB***

This document is intended as an overview of the information provided and also as an explanation of how the given supporting information addresses the materials that has been requested for the review of the hESC lines SA001, SA002 and SA002.5 for use in NIH Funded Research.

While the consent forms alone does not in writing contain all the elements listed in Section IIA of July 7, 2009 NIH Guidelines on Human Stem Cell Research. Additional procedures at the Sahlgrenska University Hospital and assurance of those procedures certify all elements 1-15 listed in Section IIA. This is further supported by documents 2-10 submitted herein.

Document 2: ***Ethical approval from the IRB***

The derivation of the SA001 and SA002 hESC lines was conducted with the approval of the Gothenburg University medical faculty research ethical committee.

This research project was originally approved December 20, 2000 (approval protocol # Ö 507-00) for a culture period of the hESC lines of six months.

The research project was then re-approved September 17, 2001 allowing an extension of the research for an additional two years (extension of approval protocol # Ö 507-00).

In February 17, 2003 the research project was re-approved again for an additional two years (approval protocol # Ö 026-03).

April 14, 2004 the regional ethics review board in Gothenburg approved a complementary application replacing the former applications (Approval protocol #Dnr 067-04), where as a harmonization step for the handling of human embryonic stem cells the earlier time restriction for the cultivation of lines established before June 2003 was deleted.

The last IRB approval from 2004 serves as a good overview and a summary of the approvals and is also the one that is now applicable for line SA001 and SA002. Therefore this document is submitted to show that the lines were established under IRB approval (right after the Swedish text follows an English translation), document 2.

Document 3: ***Donor consents***

All the IRB approvals, as listed above, were accompanied by updated donor consents. The donating couple of the material for line SA001 and SA002 were asked for extension of the culture time and eventually for a deletion of the time restriction.

Appendix i: The donor consent accompanying approval protocol # Ö 507-00;
Appendix ii: The donor consent accompanying the extension of approval protocol # Ö 507-00;
Appendix iii: The donor consent accompanying approval protocol # Ö 026-03;
Appendix iv: The donor consent accompanying approval protocol #Dnr. 067-04.

Document 4: ***Assurance of signing of consent for human Embryonic Stem Cell line SA001, SA002 and SA002.5***

This document is provided to assure that that informed consent from the donating couples have been gained.

Document 5: ***Assurance of conditions of consent for human Embryonic Stem Cell line SA001, SA002 and SA002.5***

A letter of assurance was obtained from the physician responsible for the stem cells project under the IRB from 2004 certifying that consent from the embryo donors whose embryos resulted in derivation of hESC line SA001 and SA002 where obtained.

In addition to the other information, this document provides supporting information demonstrating that the SA001 and SA002 hESC line was derived from human embryos:

1. that were created using in vitro fertilization for reproductive purposes and were no longer needed for this purpose; and
2. that were donated by donor(s) who gave voluntary written consent for the human embryos to be used for research purposes.

It also provides a written assurance that the principles articulated in Section IIA of the July, 2009 NIH Guidelines on Human Stem Cell Research as well as the HHS regulations for the Protection of Human Research Subjects (45 C.F R. 46 Subpart A) were followed.

Additionally this document provides a written assurance that during the informed consent process (written and oral) that the donor(s):

1. were informed of other available options pertaining to the use of the embryos;
2. would not be offered any inducements for the donation of the embryos; and
3. were informed about what would happen to the embryo after the donation for research.

This document should therefore be considered as a demonstration that the derivation of the SA001 and SA002 hESC line was conducted in accordance with all the eligibility requirements specified in Section IIB of the July, 2009 NIH Guidelines on Human Stem Cell Research.

Document 6: Anonymity process for the SA001 and SA002 human embryonic stem cell lines

In November 2006 the regional ethics review board in Gothenburg approved the request from Sahlgrenska University Hospital to make of line SA001 and SA002 anonymous. This process was also accompanied by an information letter to the donors and a documentation of that this took place.

The enclosed documents (in document 6) in Swedish are all followed by translations in English:

1. "Angående avidentifiering av humana embryonala stamcellslinjer"/"Regarding process of making human embryonic stem cell lines anonymous
2. SA002 - "Avidentifiering av humana embryonala stamcells linjer"/ "Anonymity process for human embryonic stem cell lines
3. SA001 - "Avidentifiering av humana embryonala stamcells linjer"/ "Anonymity process for human embryonic stem cell lines
4. "Information angående avidentifiering av donerat material"/"Information regarding anonymity of donation material"

Document 7: Information about human embryonic stem cell line SA001

This document is provided to give an overview regarding the characterization of cell line SA001 and furthermore to confirm the embryo source being frozen IVF surplus material, (see "Summary of characteristics of LOT AK001"). Regarding the sequence of events for a given donor(s) during the time of donation, the date of first signed informed consent was March 6, 2001 for SA001, see document 4. The cell line was established March 20, 2001, see "Summary of characteristics of LOT AK001" document 7 and document 4.

Document 8: Information about human embryonic stem cell line SA002 and SA002.5

This document is provided to give an overview regarding the characterization of cell line SA002 and SA002.5 (subclone of SA002) and furthermore to confirm the embryo source being frozen IVF surplus material, (see "Summary of characteristics of LOT AL002"). Regarding the sequence of events for a given donor(s) during the time of donation, the date of first signed informed consent was May 6, 2001 for SA002, see document 4. The cell line was established May 21, 2001, see "Summary of characteristics of LOT AL002", document 8 and document 4. The information regarding the time line for the donation and the establishment is the same for cell line and subclone SA00.5 as for its mother line SA002.

Document 9: Heins - Stem Cells. 2004;22(3):367-76.

The derivation of the SA002 hESC line, along with six additional hESC lines was published in 2004. This to provide as an example for the establishment process of hESC lines at Cellartis at this time.

Heins N, Englund MC, Sjöblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H., Derivation, characterization, and differentiation of human embryonic stem cells., Stem Cells. 2004;22(3):367-76.

Document 10: Sample Letter of SO

Translation of application to the regional ethics review board in Gothenburg.
Dr. Boo Edgar, 2005-03-23

The regional ethics review board in Gothenburg
Att Inger Hellström
Dept Internal Medicine
Box 454
405 30 Göteborg

Regarding addition to the ethics applications 2000-11-20 # 507-00, extension for 2 years (2001-09-17, # Ö507-00,) Ö026-03(additional extension for 2 years) Ö025-03 and S454-03

The stem cell research at the medical faculty at, Sahlgrenska Academy, was initiated through an Ethics application autumn 2000 (2000-11-20, #507-00).The application was approved, but with an limitation that the stem cells should only be grown for 6 months. This has later been extended through additionally approved applications on 2 years extension (Ö507-00 and Ö026-03). Under these ethics applications have 20 embryonic stem cell lines been established. These lines are traceable to the donors through a special registry, which is administered by the dept of obstetric and gynaecology.

With the ethics permission 2003-05-19 (# Ö025-03) the establishment of non-traceable stem cells where additional patient approvals may not be given. These cells may also be sent abroad to other researchers.

In 2001-02-13 the ethics research committee at Uppsala University permitted the establishment of human embryonic stem cells without a time restriction (Ups00-536).

Through an additional decision 2003-10-21 (#S454-03) it was permitted that cells could be sent to Canada as part of a research collaboration without additional informed consent by the patients.

Under 2003 we have been asked by several collaborative researchers, and we are contractually bound to deliver human embryonic stem cell lines to the NIH registry and researchers attached to the Juvenile Diabetes Research foundation. As the donors are increasingly frustrated of signing informed consents, being seen to be identical with earlier documents, and that we researchers consider it to be illogical that different stem cell lines have different ethical principals for there use we would like to harmonise the handling.

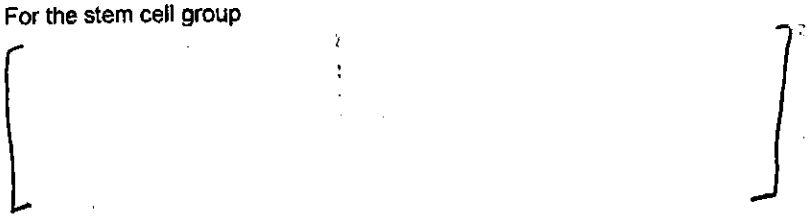
The routines for the established cell lines should be compared to other cell lines of human origin that are used in research across the world, for instance tumour cell lines from ATCC . The practical process is that this letter and the informed consent form the addition to the ethics approvals as above. Some of the cell lines will be traceable from practical reasons, the NIH lines when it is necessary. Restrictions for other researchers are govern by the law in the respective country and one of the Sahlgrens Academy approved 'materials transfer agreement' where the receiving research group cannot send the cell lines to another group, sell the cell line and that we may ask the group to return the cell-line should the Swedish law demands that.

Gothenburg February 3rd 2004

Christina Bergh
Professor
Reproductive medicine
SU/Sahlgrenska

Lars Nilsson
Docent Head Physician
Reproductive Medicine
SU Sahlgrenska

For the stem cell group



Translation of decision of the regional ethics review board in Gothenburg.
Dr. Boo Edgar, 2005-03-23

Professor Anders Lindahl
Dept of Clinical Chemistry/Transfusion Medicine
SU/Sahlgrenska
413 45 Göteborg

Göteborg 2004-04-14
BR/ih

Let it be known that the application Dnr 067-04 adds to/replace the earlier application Dnr Ö 507-00 and Ö 026-03.

As a harmonization step for the handling of human embryonic stem cells are the earlier time restriction for the cultivation of lines established before June 2003 deleted. This should also be noted in a special remark in the patient information to concerned donors.

Best regards,

Bo Risberg
Professor
Academic Secretary

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January 8, 2009

Concerning research on human embryonic stem cells established at Sahlgrenska University Hospital:

Informed consent from the donating couples have been gained for stem cell line SA002 and SA001 were the IRB approval concerning the human embryonic stem cell lines applies (Dnr 067-04; Records from meeting April 14, 2004). SA002.5 is a subclone of SA002 and is therefore covered by the donor consent of SA002.

Summary of Donor Consent Details

Cell line nr.	Ethical approval from IRB	Establishment date	Date of first signed informed consent	Date of last signed informed consent	Boxes ticked in the last signed donor consent form		
					We permit that these stem cells are sent abroad for research purposes.	We do not permit that these stem cells are sent abroad for research purposes.	We wish to be contacted prior to any new research purpose.
SA001	Ö 507-00, Ö026-03, 067-04	2001-03-20	2001-03-06	2004-12-06	Yes	No	No
SA002	Ö 507-00, Ö026-03, 067-04	2001-05-21	2001-05-06	2004-11-27	Yes	No	No

Christina Bergh

Professor
Reproductive Medicine
Department of Obstetrics and Gynaecology
Institution of Clinical Sciences, Sahlgrenska Academy
Sahlgrenska University Hospital
S-413 45 GÖTEBORG
Sweden

Appendix 1 (English) (17)

This is a translation from the Swedish original (Ö507-00) presented to the donor couple.

Sahlgrenska University Hospital
Division of Women's Healthcare, Urology and Oncology 2000-12-04 eaf

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Patient information

Regarding human embryos and the culture of stem cells

In vitro fertilization (IVF) normally results in supernumerary fertilized eggs (embryos), i.e. more eggs than what can be transferred back to the woman at the time of treatment are obtained. If those embryos are considered normal after a microscopic inspection, they are frozen and can be transferred back to the woman at a later point of time if so desired. If the fertilized eggs are of low quality and it is concluded that they are either not suitable for transfer to the woman or that they would not survive the freezing and thawing procedure, they are currently destroyed. Those embryos can however still be useful in a research context. Some of those embryos can be maintained in culture for 5-6 days, and if cells are isolated from those cultured fertilized eggs, those cells can develop into a variety of cell types, like nerve cells, muscle cells or different types of blood corpuscles. We believe that in the future this will be an important way of producing cells that at a later stage can be transplanted to patients suffering from injuries after accidents (spinal cord injuries, joint injuries) or diseases such as heart attacks.
In the current project we will only develop techniques making long term culture available of those otherwise destroyed cells. The cells will not be used for any clinical purpose. The cells will be destroyed after the completion of this study. Your participation in this project is of course totally voluntary, and if you decide not to participate this will not under any circumstances affect the medical care given to you. You can, at any time, and without further explanation, terminate your participation

Further information can be gained form.

Lars Nilsson
Associated Professor, Chief Physician
Phone: 031-342 1000 ext. 7893

Christina Bergh
Associate Professor, Care Unit Chief Physician
Phone: 031-342 1000 ext. 7452

[ ] was taking the initiative to this study and he will also be happy to answer to questions relating to embryonic stem cells, the risks and possibilities of those for the future. Telephone: [ ]

Women's Health Care
Sahlgrenska University Hospital/Sahlgrenska
[ ] [ ]

Telephone: [ ]
Fax: [ ]

Culture of stem cells from human embryos

I have been informed orally about the study and have read the above written information. I am aware that my participation in the study is completely voluntary and that I can terminate my participation at any time without explanation and this will not affect my care taking.

Date

Name ....

Name (Print)

Kvinnosjukvården
Sahlgrenska University Hospital / Sahlgrenska
[ ] GOTHENBURG
Phone: [ ]
Fax: [ ]
email: [ ]

This is a translation from the Swedish original (Ö507-00) presented to the donor couple.

Sahlgrenska University Hospital
Division of Women's Healthcare, Urology and Oncology

Regarding human embryos and the culture of stem cells

In connection to your IVF treatment (date to be inserted).....you donated supernumerary embryos for stem cell research. The information you then received stated that the cells would be destroyed after about 6 months in culture. This time is now approaching. However we wish to continue the culture of those cells to get a better understanding of the stability of the cells during long time culture. We would hereby like to ask if you approve those cells to be cultured for an additional two years. We are grateful for your return of this form after signing.

We permit that our cells are cultured up to 2 years ....

We do not permit that our cells are cultured more than 6 months. ....

(Please, mark the selected alternative with a cross)

Further information can be obtained form.

Lars Nilsson
Associated Professor, Chief Physician
Phone: 031-342 1000 ext. 7893

Christina Bergh
Associate Professor, Care Unit Chief Physician
Phone: 031-342 1000 ext. 7452

[ ] was taking the initiative to this study and he will also be happy to answer to questions relating to embryonic stem cells, the risks and possibilities of those for the future. Telephone: [ ]

I have been verbally informed about the study and I have read and understood the above written information. I am aware of that my participation in this study is totally voluntarily and that I can, at any time, and without further explanation, terminate my participation, without affecting the medical care given to me.

Date

Signature..... .....

Clarification of signature

Women's Health Care
Sahlgrenska University Hospital/Sahlgrenska
[ ] Gothenburg

Telephone: [ ]
Fax: [ ]
email: [ ]

Appendix iii

This is a translation from the Swedish original presented to the donor couple.

Women's Healthcare, Sahlgrenska University Hospital
Field of activities; Reproductive Medicine

Regarding human embryos and the culture of stem cells

In connection to your IVF treatment in 2001, you donated supernumerary embryos for research purposes in the field of stem cell research. The information you then received stated that the cells would be destroyed after about 2 years in culture. This time is now approaching. However we wish to continue culturing of those cells to get a better understanding of the cells stability and their ability to differentiate to different cell types at long time culture. Continuous handling of the cells will be carried out according to Swedish law and the governmental decisions such as the new law on biobanking. We would hereby like to ask if you approve those cells to be cultured in additional up to 2 years. Grateful for your return of this form upon signing.

See p. 67 for translation correction

We permit that our cells are cultured up to 2 years ....

We do not permit that our cells are cultured more than 2 years ....

(please, mark the selected alternative with a cross)

Further information can be gained form.

Christina Bergh
Professor, Care Unit Chief Physician
Phone: 031-342 23 75

Lars Nilsson
Associated Professor, Chief Physician
Phone: 031-342 13 23

[ ] was taking the initiative to this study and he will also be happy to answer to questions relating to embryonic stem cells, the risks and possibilities of those for the future. Telephone: [ ]

I have been verbally informed about the study and I have read and understood the above written information. I am aware of that my participation in this study is totally voluntarily and that I can, at any time, and without further explanation, terminate my participation, without affecting the medical care given to me.

Date
Donor 1 ..... Donor 2 .....
Clarification of signature

Women's Health Care
Sahlgrenska University Hospital/Sahlgrenska
[ ] Gothenburg
Telephone: [ ]
Fax: [ ]
email: [ ]

This is a translation from the Swedish original presented to the donor couple.
Translated by Mikael Englund, PhD, 2004-10-06

Women's Healthcare, Sahlgrenska University Hospital
Field of activities; Reproductive Medicine

Patient information

Regarding human embryos and the culture of stem cells

In connection to your IVF treatment in 2001, you donated supernumerary embryos for research purposes in the field of stem cell research. The results that have been generated to date or that will be generated in the future as a result of this research on these embryos, for example stem cells, might be used in further research and development of pharmaceutical drugs and methods of treatment of severe widespread diseases. This research includes the use of stem cells for analyses of metabolism and toxicity, as well as directing stem cells towards more mature cells like neural tissue, connective tissue, heart and liver cells, and insulin producing cells. The aim is that such research shall make it possible to treat, and hopefully cure, patients with severe diseases like heart infarction and diabetes, or patients subjected to severe accidents that have generated spinal cord-, or joint injuries.

This research does not only take place in Sweden, but also internationally. We now wish to share the stem cells that have been developed through research on your donated embryos, to stem cell research groups in other countries.

The progressing handling of the stem cells will be in accordance to Swedish law. The cells will be cultured for the time being, and can not be traced back to you by any person not directly concerned, while the key to the code is only available at the Institution for Women's and Children's Health. We hereby ask you if you permit that the stem cells we have developed from the supernumerary embryos you have donated are sent to research groups abroad for the research purposes.

We are grateful if you resend this form after signing.

SAHLGRENSKA UNIVERSITY HOSPITAL
FIELD OF ACTIVITIES; REPRODUCTIVE MEDICINE

Christina Bergh
Professor, Care Unit Chief Physician
Phone: 031-342 23 75

Lars Nilsson
Associated Professor, Chief Physician
Phone: 031-342 13 23

I have been verbally informed about the study and I have read and understood the above written information. I am aware of that my participation in this study is totally voluntarily and that I can, at any time, and without further explanation, terminate my participation, without affecting the medical care given to me.

We permit that these stem cells are sent abroad for research purposes. ☐

We do not permit that these stem cells are sent abroad for research purposes. ☐

We wish to be contacted prior to any new research purpose. ☐

Date.....

Signature.....

Clarification of signature..... social security number.....

Signature.....

Clarification of signature..... social security number.....

This is a translation from the Swedish original letter from the responsible physician to the regional institutional review board (IRB). The letter was reviewed and Approved by the IRB November 13, 2006.

Gothenburg, 7 November, 2006

Bo Risberg, Professor, Scientific Secretary

Regional Ethical Review Board in Gothenburg
Department of Medicine, Box 454
405 30 Gothenburg

Approval stamp: Reviewed and approved as a secretary matter. 2006-11-13
Bo Risberg, professor, Scientific Secretary
Regional Ethical Review Board in Gothenburg

Regarding process of making human embryonic stem cell lines anonymous

The human embryonic stem cell lines, established after the 16th of June 2006, in the collaboration between Sahlgrenska University Hospital (SU) and Cellartis were made anonymous already at the time of establishment, while the stem cell lines that were established before this date are traceable. This means that the traceable lines can be traced to the donating couple by linking three separate databases.

Due to the changes in the Swedish law enabling broader work on human embryonic stem cell lines made anonymous in comparison to the traceable we wish to make some of those traceable lines anonymous, i.e. erase the link to the donating couple in the database. Making the lines anonymous would considerably simplify the handling surrounding the stem cell lines and enable research also for big pharma and biotech companies, which permits new doors for the stem cell research to be opened.

At a meeting between the department of Reproductive medicine (SU), Cellartis and Professor Bo Risberg on the 18th of April, 2006, an initiation of the process of making the stem cell lines anonymous was discussed. The details regarding the practical handling of this process have now been chosen and are described in the enclosed, "*Anonymity process for human embryonic stem cell lines*".

The donating couple will be informed by letter that a total anonymity process has been carried out.

The documents for the process of making the stem cell lines anonymous will be signed by the laboratory manager at the IVF clinic, practically carrying out the process, by the Chief Scientific Officer at Cellartis AB and by the responsible physician for the stem cell project at Sahlgrenska University Hospital.

Best regards,

Christina Bergh, MD, Professor

Department of Obstetrics and Gynaecology
Sahlgrenska University Hospital

Gothenburg 5th of June, 2007

Anonymity process for human embryonic stem cell lines

The stem cells lines established in the collaboration between Sahlgrenska University Hospital (SU) and Cellartis after the 16th of June, 2003 are anonymous, i.e. the material cannot be traced back to the donating couple. The stem cell lines established before this date are traceable and could by linking three separate databases be traced back to the donors. Due to a change in the Swedish law today allowing a broader use for non-traceable lines compared to traceable[1,2] we hereby aim to make some of those traceable lines anonymous, i.e. erase the link to the donating couple. This will considerable simplify the handling surrounding the stem cell lines and enable research also for pharmaceutical and biotech companies, which increases the possibilities for stem cell research.

Data storage of traceable stem cell lines

The three different data bases where information regarding the establishment of the stem cell lines is stored are; the patient file, the IVF-treatment registry number, and the Blastocyst registry for research, figure 1.

Data base I is only accessible to SU personnel that are not directly connected to the stem cell project. On this data base the patient data is connected to the patient treatment number (IVF-treatment registry number) If the donated fertilized eggs reaches the blastcyst stage (day 5-7 after fertilization) the are given a blastocyst number. At this stage the blastocysts are moved physically to another building for continuous culture.

In ***Data base II*** the IVF-number is linked to a blastocyst number. Here information regarding the culture conditions and the results from the culture of the fertilized eggs are stored. No information regarding the patient or information needed for the IVF treatment is stored here. Only two persons have access to this data base, Dr

[redacted]

If the establishment of a stem cell line is successful the blastocyst number also becomes the number of the stem cell line, this information is stored in ***data base III***, which is physically located at Cellartis AB. The persons having access to data base III do not have access to data base II and can therefore not trace the patient information.

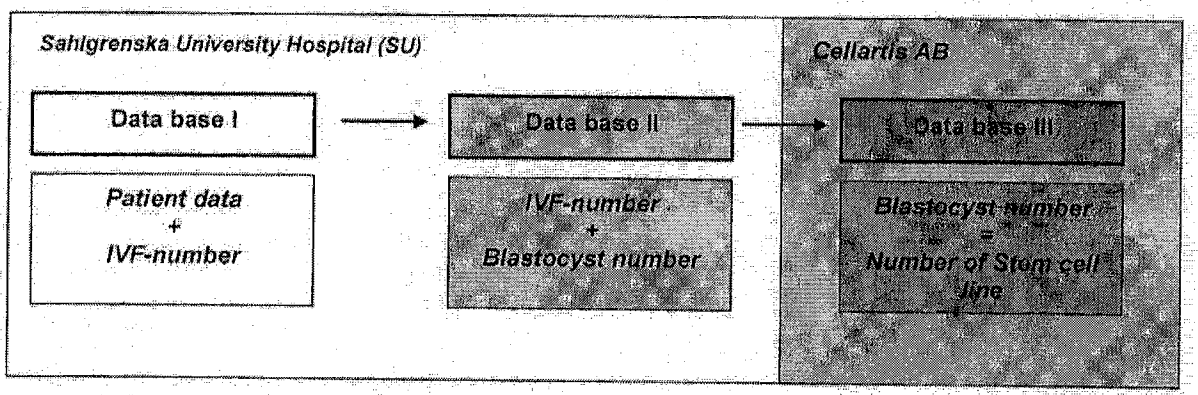

Figure 1, *Description of how patient information, IVF-number och blastocyst number is stored in three separate data bases. The personnel having authorization to access data base III do not have access to data base II and can therefore not trace patient information.*

[1] Prop. 2005/06:64, Genetisk Integritet (Sveriges Riksdag)/Genetic Integrity (Swedish Government)
[2] Prop. 2003/04:148 Stamcellsforskning (Sveriges Riksdag)/ Stem Cell Research (Swedish Government)

Making the traceable stem cell lines anonymous

To make the the traceable stem cell lines anonymous, it is required that the link between data base I and data base II is erased. This is done by erasing the IVF-number from data base II. Only two persons, Dr Kersti Lundin and Mrs Anita Sjögren, have access to this data base. The erasing will be carried out by those two persons together.

In concordance with existing law Lars Nilsson confirms, as the only person that has been in direct contact with the donor couple that the matter is now under secrecy.

Making Stem Cell Line SA001 Anonymous

Stem cell line SA001, have the _____, 2007 been made anonymous by erasing of the IVF-number from data base II, according to the process described above.

The donating couple has by letter been informed that the stem cell line will be made anonymous.

_____ _____

Name and employment: Date, place

[redacted]

Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place

[redacted]

Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place
Lars Nilsson, MD
Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place
Christina Bergh, MD, professor,
Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

(26)

Gothenburg 30th of January, 2007

Anonymity process for human embryonic stem cell lines

The stem cells lines established in the collaboration between Sahlgrenska University Hospital (SU) and Cellartis after the 16th of June, 2003 are anonymous, i.e. the material cannot be traced back to the donating couple. The stem cell lines established before this date are traceable and could by linking three separate databases be traced back to the donors. Due to a change in the Swedish law today allowing a broader use for non-traceable lines compared to traceable[1] [2] we hereby aim to make some of those traceable lines anonymous, i.e. erase the link to the donating couple. This will considerable simplify the handling surrounding the stem cell lines and enable research also for pharmaceutical and biotech companies, which increases the possibilities for stem cell research.

Data storage of traceable stem cell lines
The three different data bases where information regarding the establishment of the stem cell lines is stored are; the patient file, the IVF-treatment registry number, and the Blastocyst registry for research, figure 1.

Data base I is only accessible to SU personnel that are not directly connected to the stem cell project. On this data base the patient data is connected to the patient treatment number (IVF-treatment registry number). If the donated fertilized eggs reaches the blastcyst stage (day 5-7 after fertilization) the are given a blastocyst number. At this stage the blastocysts are moved physically to another building for continuous culture.

In ***Data base II*** the IVF-number is linked to a blastocyst number. Here information regarding the culture conditions and the results from the culture of the fertilized eggs are stored. No information regarding the patient or information needed for the IVF treatment is stored here. Only two persons have access to this data base, Dr

[redacted]

If the establishment of a stem cell line is successful the blastocyst number also becomes the number of the stem cell line, this information is stored in ***data base III***, which is physically located at Cellartis AB. The persons having access to data base III do not have access to data base II and can therefore not trace the patient information.

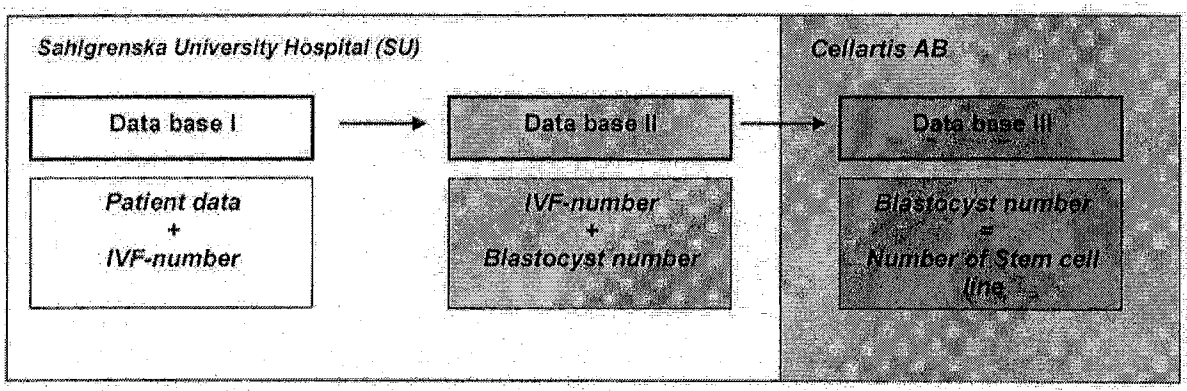

Figure 1, *Description of how patient information, IVF-number och blastocyst number is stored in three separate data bases. The personnel having authorization to access data base III do not have access to data base II and can therefore not trace patient information.*

[1] Prop. 2005/06:64, Genetisk Integritet (Sveriges Riksdag)/Genetic Integrity (Swedish Government)
[2] Prop. 2003/04:148 Stamcellsforskning (Sveriges Riksdag)/ Stem Cell Research (Swedish Government)

Making the traceable stem cell lines anonymous

To make the the traceable stem cell lines anonymous, it is required that the link between data base I and data base II is erased. This is done by erasing the IVF-number from data base II. Only two persons, Dr Kersti Lundin and Mrs Anita Sjögren, have access to this data base. The erasing will be carried out by those two persons together.

In concordance with existing law Lars Nilsson confirms, as the only person that has been in direct contact with the donor couple that the matter is now under secrecy.

Making Stem Cell Line SA002 anonymous

Stem cell line SA002, have the _____, 2007 been made anonymous by erasing of the IVF-number from data base II, according to the process described above.

The donating couple has by letter been informed that the stem cell line will be made anonymous.

_____ _____

Name and employment: Date, place

Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place

Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place
Lars Nilsson, MD
Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

_____ _____

Name and employment: Date, place
Christina Bergh, MD, professor,
Department of Obstretrics and Gynaecology
Sahlgrenska University Hospital

28

Women's Health Care, Sahlgrenska
Department of Obstetrics and Gynaecology/Reproductive Medicine

Gothenburg, date month, year

Information regarding anonymity of donated material

Dear patient,

The results so far achieved within stem cell research looks very promising and we hope that we can continue this research with the best of resources. Due to the changes in the Swedish law enabling broader work on non-identifiable human embryonic stem cell lines in comparison to material possible to trace, we will remove any links to the donors identities, i.e. erase the link to you that has only been available at the Institution for Clinical Sciences and which has previously linked you to the stem cell line.

With this Letter of Information we wish to inform you that the material donated in connection to your IVF treatment 2001 will now be made totally anonymous. This process means the donated material can never be traced back to you via the databases at the Sahlgrenska University Hospital.

The research within the stem cell field will continue in the same direction as earlier and the results achieved this far as well as future results achieved through the academic and the commercial research activities can subsequently be used in coming research and development of drug and treatment methods of serious diseases. The research comprises the use of stem cells for metabolism and toxicity studies and the directed differentiation of stem cells into more mature cells such as neuronal tissue, connective tissue, heart cells, liver cells and insulin producing cells. The aim of this research is to enable treatment of important pathological conditions such as heart attack and diabetes or serious accidents including spinal cord and joint injuries.

We thank you for your collaboration. If you have any further questions or enquiries please contact Lars Nilsson.

Best Regards,

Lars Nilsson, MD

Department of Obstetrics and Gynaecology,
Sahlgrenska University Hospital

Tfn: 031-342 13 23

Cellartis AB
Arvid Wallgrens Backe 20
SE-413 46 Göteborg, SWEDEN

Human embryonic stem cell line SA001, LOT AK001

Background

For the purpose of *in vitro* fertilization (IVF) treatment of patients suffering from involuntary childlessness, human embryos are created at the IVF clinics using conventional IVF-techniques. Supernumerary embryos may, after written informed consent from the donors, be used for research purposes, such as for derivation of human embryonic stem cells (hESC). The hESC derivation process at the University of Göteborg, Sweden and Cellartis follows all applicable laws in Sweden and is approved by the Local Research Ethics Committees at Göteborg University and Uppsala University.

Donor confidentiality

In order to protect the privacy and the confidentiality of the donors, all identifiers associated with the embryo donors have been removed. Thus, no information about the donors is accessible. Notably, the donation did not result in any financial gain for the donors.

Summary of characteristics of LOT AK001

Parameter	Passage	Result
Embryo source	--	Frozen, surplus from IVF
hESC line derived	--	March 20, 2001
Procedure for isolation of ICM cells	--	Immunosurgery
LOT preparation	p13	>100 vials
Thawing recovery rate	p13 – p14	100 %
SSEA-1	p31	Negative
SSEA-3	p31	Positive
SSEA-4	p31	Positive
TRA-1-60	p31	Positive
TRA-1-81	p31	Positive
Oct-4	p31	Positive
Alkaline phosphatase	p31	Positive
Karyotype	p52	46, XY
FISH (X, Y, 13, 18, and 21)	p32, p51	Diploid, XY
FISH (12 and 17)	p51	Diploid
MLPA (sub-telomeric regions)	p26, p49	No remark
Telomerase activity	p27	Positive
Pluripotency i*n vitro*	p29, p45	Endo-, ecto-, mesoderm
Pluripotency i*n vivo*	p19	Endo-, ecto-, mesoderm
Mycoplasma	p24	Negative
Human Immunodeficiency Virus type 1 and 2	P13	Negative
Hepatitis B	P13	Negative
Hepatitis C	P13	Negative
Cytomegalovirus	p25	Negative
Herpes Simplex Virus type 1 and 2	p25	Negative
Epstein-Barr Virus	p25	Negative
Human Papilloma Virus	p25	Negative

Cellartis AB
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Details

Derivation of hESC line SA001

Establishment of hESC lines at Cellartis is performed according to the procedures described in Heins *et al.* (Stem Cells, May 2004) and in Patent application "A method for the establishment of a pluripotent human blastocyst-derived stem cell line" (PCT no. PCT/EP02/14895, Publication no. WO03/055992). For routine expansion, the hESC are cultured on top of a mouse embryonic feeder (mEF) layer using VitroHES™ medium provided by Vitrolife AB (Göteborg, Sweden).

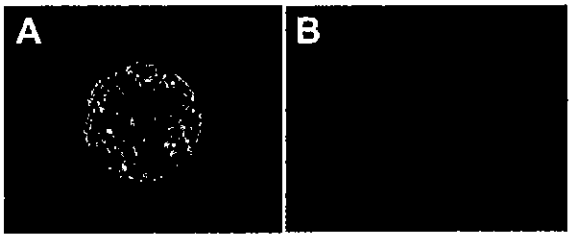

Figure 1. (A) Blastocyst from which hESC line SA001 was derived. (B) After Immunosurgery and plating on mEF.

Morphology

At the time of vitrification >100 vials were prepared from the hESC line SA001 in passage 13. Typical morphology of the hESC colonies, just prior to vitrification, is shown in Figure 2. After thawing and seeding of vitrified cells (*i.e.* LOT AK001), viable colonies proliferated and displayed the morphology that characterizes undifferentiated hESC (Figure 3). Subsequently, these cells were propagated and passaged according to standard procedures and representative illustrations of the hESC colonies in passage 18, 23, 29, and 35 are shown in Figure 4.

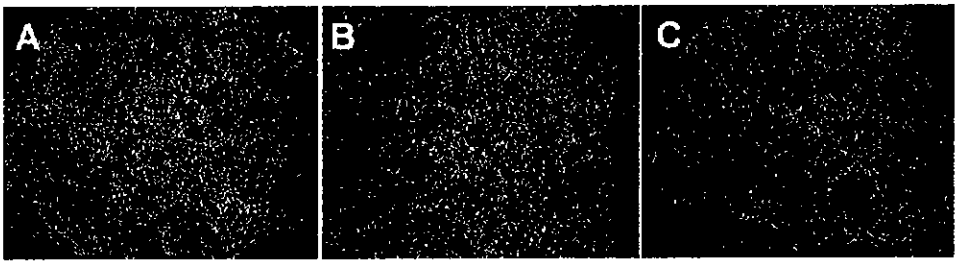

Figure 2 (A)-(C). Typical morphology of hESC line SA001 cultured on mEF in passage 13 just prior to vitrification.

Figure 3 (A)-(C). Typical morphology of hESC cultured on mEF (passage 14) after thawing of vitrified cells (LOT AK001).

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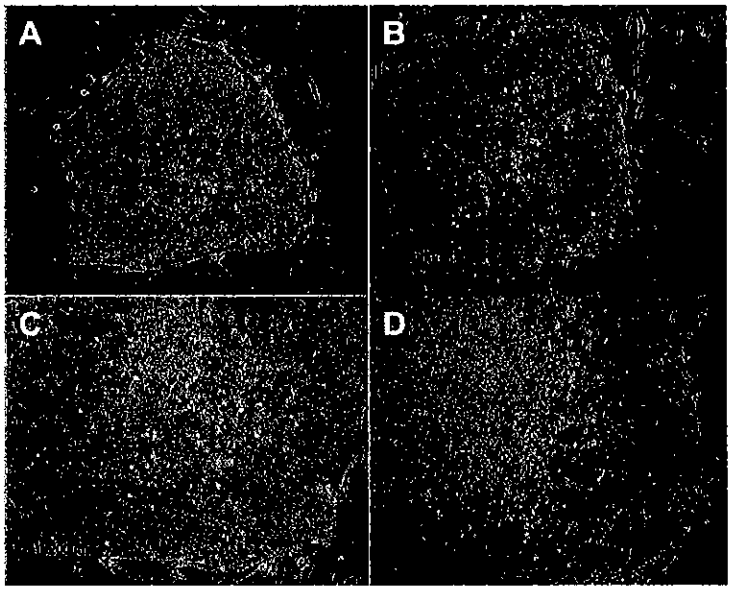

Figure 4. Typical morphology of hESC of LOT AK001 cultured on mEF in passage 18 (A), passage 23 (B), passage 29 (C), and passage 35 (D).

Thawing recovery rate

The viability of hESC LOT AK001 was determined by measuring the thawing recovery rate. Briefly, out of the >100 frozen vials of LOT AK001, ten vials were sampled, thawed, and seeded in ten separate dishes containing mEF and VitroHES™ medium. The number of hESC clumps that were seeded, attached, proliferated, and displayed appropriate morphology was determined for each dish. The results are presented in Figure 5 and show that all ten vials (100 %) gave rise to viable hESC colonies. These cells were subsequently passaged according to standard procedures and used for the characterization presented in this document.

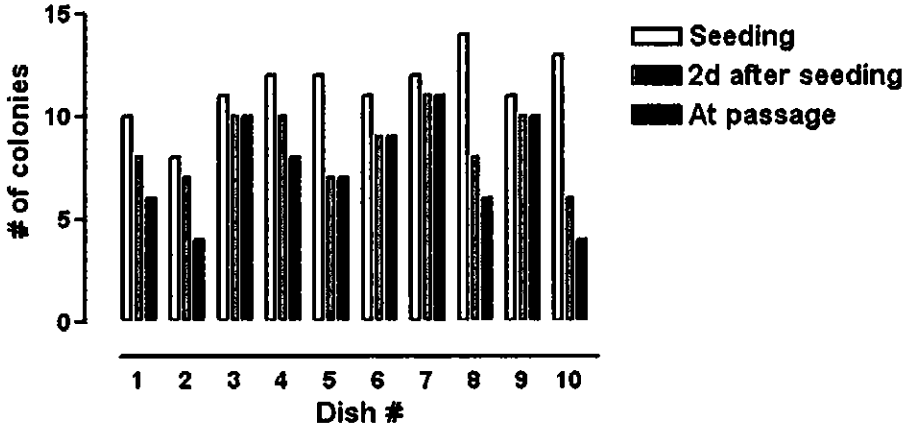

Figure 5. Thawing recovery rate of LOT AK001. Thawed hESC were seeded and the number of hESC clumps from each vial was determined (open bars) and subsequently the number of viable colonies was determined two days after seeding (grey bars) and at the time of passage (black bars).

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Immunohistochemical staining of undifferentiated hESC

Undifferentiated hESC colonies of LOT AK001 were fixed in PFA and subsequently permeabilized using Triton X-100. After consecutive washing and blocking steps, the cells were incubated with the primary antibody (as indicated in the figure legend). Conjugated secondary antibodies were subsequently used for detection. The nuclei were visualized by DAPI staining. The activity of Alkaline phosphatase (ALP) was determined using a commercial available kit following the instructions indicated by the manufacturer (Sigma Diagnostics, Stockholm, Sweden). The passage number at which each analysis was performed is indicated within brackets in the figure legend. The results show that hESC of LOT AK001 are negative for SSEA-1 (B) and positive for SSEA-3 (C), SSEA-4 (D), TRA-1-60 (E), TRA-1-81 (F), Oct-4 (G), and ALP (H).

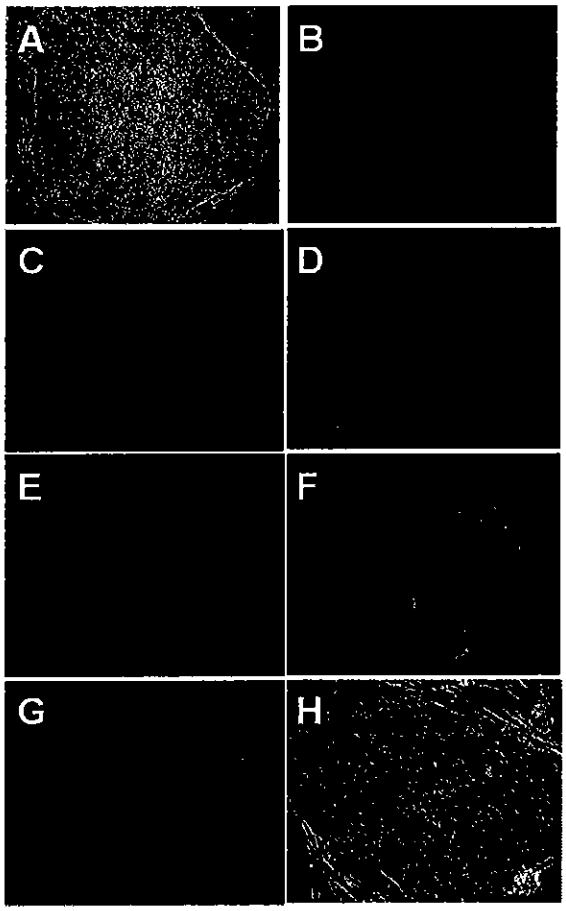

Figure 6 (right). (A) hESC colony [p19], (B) SSEA-1 [p31], (C) SSEA-3 [p31], (D) SSEA-4 [p31], (E) TRA-1-60 [p31], (F) TRA-1-81 [p31], (G) Oct-4 [p31], (H) ALP [p31]

Cellartis AB
Arvid Wallgrens Backe 20
SE-413 46 Göteborg, SWEDEN

Karyotyping

The cells were incubated in the presence of Calyculin A and then washed with cell culture medium. The cells were collected by centrifugation and fixed using ethanol and glacial acetic acid. The chromosomes were visualized using a Trypsin-Giemsa staining (Figure 7) and no abnormalities were observed.

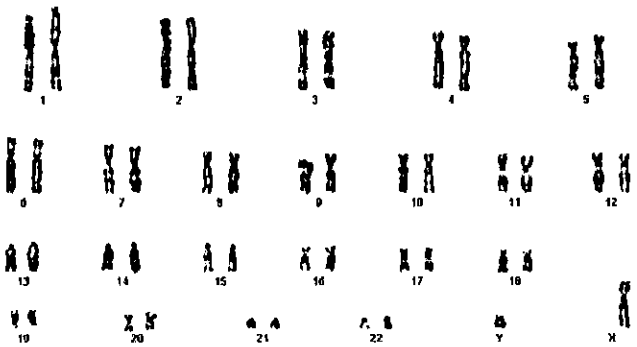

Figure 7. Karyotype of LOT AK001 in passage 26.

FISH

Probes for chromosome 12 and 17, in addition to a commercially available kit containing probes for chromosome 13, 18, 21 and the sex chromosomes (X and Y) was used following the instructions from the manufacturer (Vysis. Inc, Downers Grove, IL, USA), with minor modifications. The slides were analyzed in an invert microscope equipped with appropriate filters and software (CytoVision, Applied Imaging, Santa Clara, CA, USA). The cells were XY and diploid for chromosome 12, 13, 17, 18, and 21.

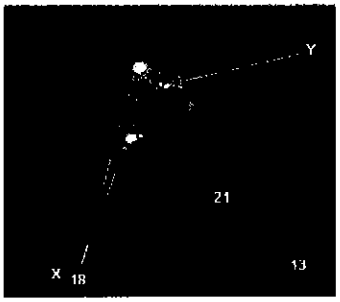

Figure 8. FISH analysis of hESC of LOT AK001 in passage 32.

Multiplex Ligation-dependent Probe Amplification (MLPA)

To detect single or multiple deletions and amplifications in the subtelomeric regions, MLPA-technology was employed using the commercially available SALSA P019/P020 Telomers MLPA kit and following the instructions provided by the manufacturer (MRC-Holland, Amsterdam, The Netherlands). The probe mixes contain in total 72 probes. One probe for each of the 48 subtelomeric regions, as well as one probe directed to a sequence in the middle of each chromosome. The analysis was performed at Department of Paediatrics, Clinical Genetics, Sahlgrenska University Hospital/ÖS using hESC of LOT AK001 in passage 26 and 49. No deletions or amplifications were detected.

Cellartis AB
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SE-413 46 Göteborg, SWEDEN

Telomerase activity

For analyzing the telomerase activity a *Tel*o TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Basel, Switzerland) was employed according to the manufacturer's instructions. The assay uses the internal activity of telomerase, amplifying the product by PCR and detecting it with an enzyme linked immunosorbent assay (ELISA). hESC of LOT AK001 were analyzed in passage 27 and displayed high telomerase activity.

Pluripotency in vitro

Undifferentiated hESC colonies were transferred to suspension cultures, using Stem Cell Cutting Tool (Swemed Lab, Göteborg, Sweden), to generate Embryoid bodies (EBs). Subsequently, these EBs were plated in tissue culture plates. Cells that spontaneously differentiated were subjected to immuno-histochemical evaluation. As illustrated in figure 9, positive staining was obtained using antibodies directed against β-III-tubulin (A), desmin (B), α-fetoprotein (C) and HNF-3β (D). Areas of spontaneously contracting cells, resembling cardiomyocytes, were also observed (not shown). Taken together, these results indicate that hESC of LOT AK001 are capable of differentiating *in vitro* to cells representing the three germ layers.

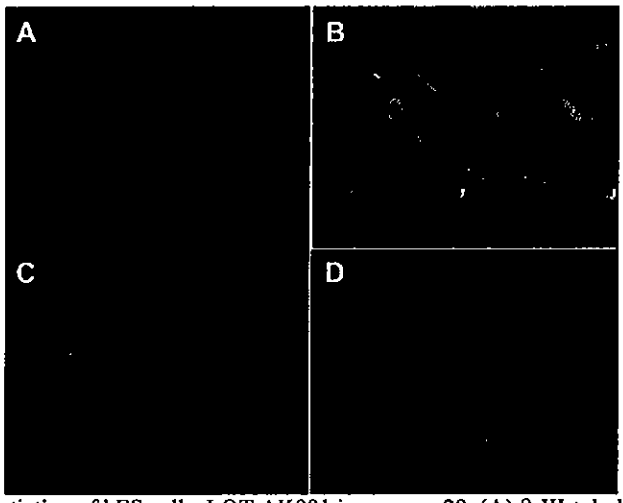

Figure 9. In vitro differentiation of hES cells, LOT AK001 in passage 29. (A) β-III-tubulin, (B) desmin, (C) α-fetoprotein, (D) HNF-3β

Pluripotency in vivo

Undifferentiated hESC were surgically placed under the kidney capsule of severe combined immuno-deficient (SCID) mice. The mice were sacrificed after 8 weeks and tumors were dissected and fixed in PFA. Histological evaluation of hematoxylin-eosin stained paraffin sections (Figure 10) demonstrated the presence of tissues derived from endo- (A), meso- (B), and ectoderm (C).

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the policy regarding the use and care of laboratory animals. All research involving animals took place at Laboratory for Experimental Biomedicine which is a specifically pathogen free, full barrier, animal facility at Göteborg University. Göteborg University has PHS Approved Animal Welfare Assurance number A5443-01.

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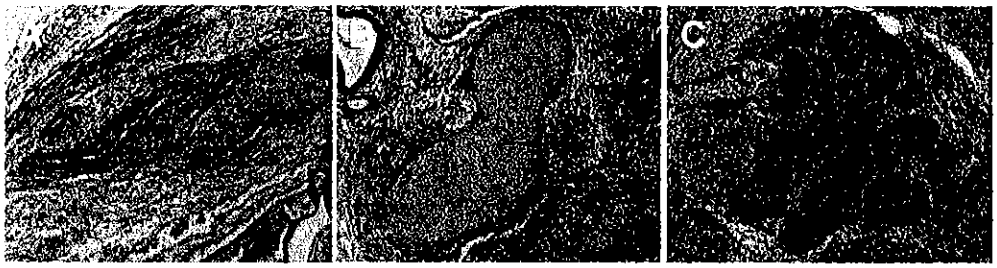

Figure 10. In vivo differentiation of hESC of LOT AK001 in passage 19. (A) Endoderm (secretory epithelium). (B) Mesoderm (cartilage). (C) Ectoderm (neuroectoderm).

Mycoplasma

The presence of mycoplasma in the hESC cultures of LOT AK001 was tested using PCR and mycoplasma specific primers. The assays were performed at the DNA Laboratory at the Department of Clinical Bacteriology, Sahlgrenska University Hospital, Göteborg, Sweden.

No mycoplasma was detected.

Human viruses

hESC of LOT AK001 were tested for the presence of Human Immunodeficiency Virus type 1 and 2, Hepatitis B, Hepatitis C, Cytomegalovirus, Herpes Simplex Virus type 1 and 2, and Epstein-Barr Virus at the Department of Clinical Virology, Sahlgrenska Academy at the University of Göteborg, Sweden. The presence of Human Papilloma Virus was analyzed at Medical Microbiology Laboratory, The University of Lund, Malmö, Sweden.

None of these viruses were detected.

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Human embryonic stem cell line SA002, LOT AL002

Background

For the purpose of *in vitro* fertilization (IVF) treatment of patients suffering from involuntary childlessness, human embryos are created at the IVF clinics using conventional IVF-techniques. Supernumerary embryos may, after written informed consent from the donors, be used for research purposes, such as for derivation of human embryonic stem cells (hESC). The hESC derivation process at Göteborg University and Cellartis follows all applicable laws in Sweden and is approved by the Local Research Ethics Committees at Göteborg University and Uppsala University, Sweden.

Donor confidentiality

In order to protect the privacy and the confidentiality of the donors, all identifiers associated with the embryo donors have been removed. Thus, no information about the donors is accessible. Notably, the donation did not result in any financial gain for the donors.

Summary of characteristics of LOT AL002

Parameter	Passage #	Result
Embryo source	--	Frozen, surplus from IVF
hESC line derived	--	May 21, 2001
Procedure for isolation of ICM cells	--	Spontaneously hatched
LOT preparation	p12	>100 vials
Thawing recovery rate	p12 – p13	100 %
SSEA-1	p14	Negative
SSEA-3	p23	Positive
SSEA-4	p23	Positive
TRA-1-60	p15	Positive
TRA-1-81	p15	Positive
Oct-4	p14	Positive
Alkaline phosphatase	p14	Positive
Karyotype	p25, p26	47, XX +13
FISH (X, Y, 13, 18, and 21)	p19	+13, XX
MLPA (sub-telomeric regions)	p14	+13, otherwise no remark
Telomerase activity	p19	Positive
Pluripotency *in vitro*	p16, p37	Endo-, ecto-, mesoderm
Pluripotency *in vivo*	p19	Endo-, ecto-, mesoderm
Mycoplasma	p23	Negative
Human Immunodeficiency Virus type 1 and 2	p15 and p28, resp.	Negative
Hepatitis B	p15	Negative
Hepatitis C	p15	Negative
Cytomegalovirus	p15	Negative
Herpes Simplex Virus type 1 and 2	p15	Negative
Epstein-Barr Virus	p15	Negative
Human Papilloma Virus	p15	Negative

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Details

Derivation of hESC line SA002

Establishment of hESC lines at Cellartis is performed according to the procedures described in Heins *et al.* (Stem Cells, May 2004) and in Patent application "A method for the establishment of a pluripotent human blastocyst-derived stem cell line" (PCT no. PCT/EP02/14895, Publication no. WO03/055992). For routine expansion, the hESC are cultured on top of a mouse embryonic feeder (MEF) layer using VitroHES™ medium provided by Vitrolife AB (Göteborg, Sweden).

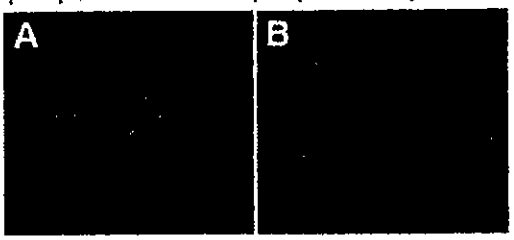

Figure 1. (A) Blastocyst from which hESC line SA002 was derived, (B) Proliferating inner cell mass cells from hatched blastocyst on MEF.

Morphology

At the time of vitrification >100 vials were prepared from the hESC line SA002 in passage 12. Typical morphology of the hESC colonies, just prior to vitrification, is shown in Figure 2. After thawing and seeding of vitrified cells (*i.e.* LOT AL002), viable colonies proliferated and displayed the morphology that characterizes undifferentiated hESC (Figure 3). Subsequently, these cells were propagated and passaged according to standard procedures and representative illustrations of the hESC colonies in passage 15, 20, 24, and 30 are shown in Figure 4.

Figure 2 (A)-(C). Typical morphology of hESC line SA002 cultured on MEF in passage 12 just prior to vitrification.

Figure 3 (A)-(C). Typical morphology of hESC cultured on MEF (passage 13) after thawing of vitrified cells (LOT AL002).

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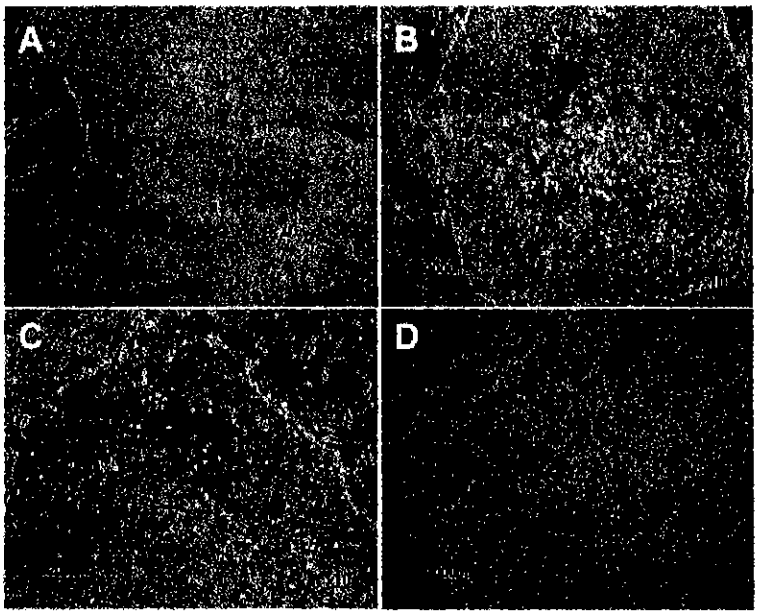

Figure 4. Typical morphology of hESC of LOT AL002 cultured on MEF in passage 15 (A), passage 20 (B), passage 24 (C), and passage 30 (D).

Thawing recovery rate

The viability of hESC LOT AL002 was determined by measuring the thawing recovery rate. Briefly, out of the >100 frozen vials of LOT AL002, ten vials were sampled, thawed, and seeded in ten separate dishes containing MEF and VitroHES™ medium. The number of hESC clumps that were seeded, attached, proliferated, and displayed appropriate morphology was determined for each dish. The results are presented in Figure 5 and show that all ten vials (100%) gave rise to viable hESC colonies. These cells were subsequently passaged according to standard procedures and used for the characterization presented in this document.

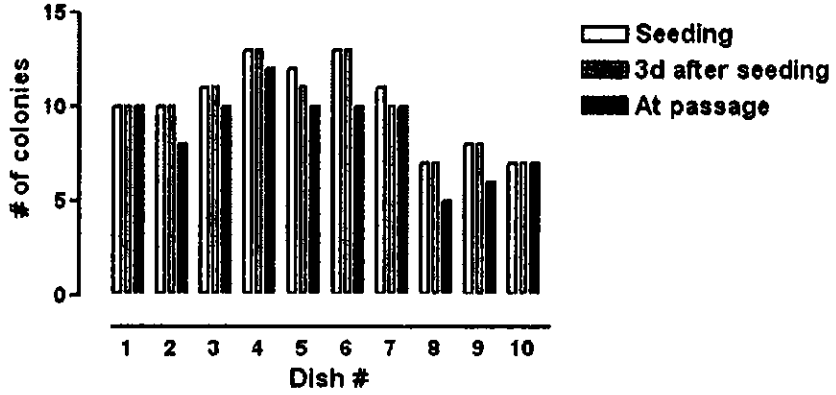

Figure 5. Thawing recovery rate of LOT AL002. Thawed hESC were seeded and the number of hESC clumps from each vial was determined (open bars) and subsequently the number of viable colonies was determined three days after seeding (grey bars) and at the time of passage (black bars).

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Immunohistochemical staining of undifferentiated hESC

Undifferentiated hESC colonies of LOT AL002 were fixed in PFA and subsequently permeabilized using Triton X-100. After consecutive washing and blocking steps, the cells were incubated with the primary antibody (as indicated in the figure legend). Conjugated secondary antibodies were subsequently used for detection. The nuclei were visualized by DAPI staining. The activity of alkaline phosphatase (ALP) was determined using a commercial available kit following the instructions indicated by the manufacturer (Sigma Diagnostics, Stockholm, Sweden). The passage number at which each analysis was performed is indicated within brackets in the figure legend. The results show that hESC of LOT AL002 are negative for SSEA-1 (B) and positive for SSEA-3 (C), SSEA-4 (D), TRA-1-60 (E), TRA-1-81 (F), Oct-4 (G), and ALP (H).

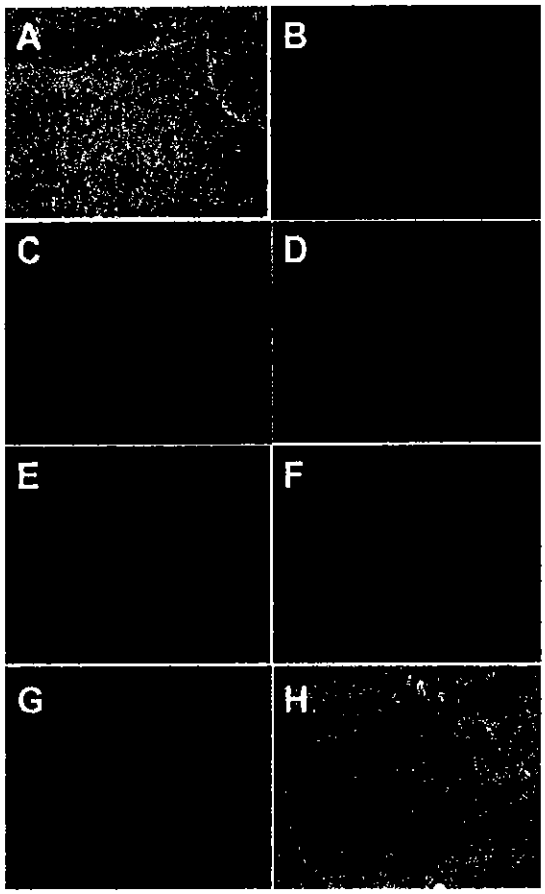

Figure 6 (right). (A) hESC colony [p29], (B) SSEA-1 [p14], (C) SSEA-3 [p23], (D) SSEA-4 [p23], (E) TRA-1-60 [p15], (F) TRA-1-81 [p15], (G) Oct-4 [p14], (H) ALP [p14]

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Karyotyping

The cells were incubated in the presence of Calyculin A and then washed with cell culture medium. The cells were collected by centrifugation and fixed using ethanol and glacial acetic acid. The chromosomes were visualized using a trypsin-Giemsa staining (Figure 7) and trisomy 13 was observed.

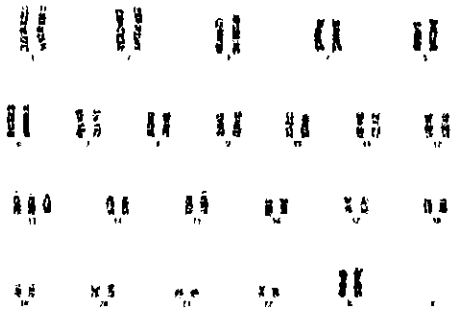

Figure 7. Karyotype of LOT AL002 in passage 26.

FISH

A commercially available kit containing probes for chromosome 13, 18, 21 and the sex chromosomes (X and Y) was used following the instructions from the manufacturer (Vysis. Inc, Downers Grove, IL, USA), with minor modifications. The slides were analyzed in an invert microscope equipped with appropriate filters and software (CytoVision, Applied Imaging, Santa Clara, CA, USA). As illustrated in figure 8, the cells were XX, diploid for chromosomes 18 and 21, and carried trisomy 13.

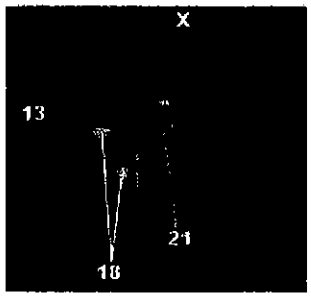

Figure 8. FISH analysis of hESC of LOT AL002 in passage 19.

Multiplex Ligation-dependent Probe Amplification (MLPA)

To detect single or multiple deletions and amplifications in the subtelomeric regions MLPA-technology was employed using the commercially available SALSA P019/P020 Telomers MLPA kit and following the instructions provided by the manufacturer (MRC-Holland, Amsterdam, The Netherlands). The probe mixes contain in total 72 probes. One probe for each of the 48 subtelomeric regions, as well as one probe directed to a sequence in the middle of each chromosome. The analysis was performed at Department of Paediatrics, Clinical Genetics, Sahlgrenska University Hospital, Göteborg, Sweden by using hESC LOT AL002 in passage 14. No deletions or amplifications were detected, except for trisomy 13.

Telomerase activity

For analyzing the telomerase activity a *Te*lo TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Basel, Switzerland) was employed according to the manufacturer's instructions. The assay uses the internal activity of telomerase, amplifying the product by PCR and detecting it with an enzyme linked immunosorbent assay (ELISA). hESC of LOT AL002 were analyzed in passage 19 and displayed high telomerase activity.

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Pluripotency in vitro

Undifferentiated hESC colonies were transferred to suspension cultures, using Stem Cell Cutting Tool (Swemed Lab, Göteborg, Sweden), to generate embryoid bodies (EB). Subsequently, these EB were plated in tissue culture plates. Cells that spontaneously differentiated were subjected to immuno-histochemical evaluation. As illustrated in figure 9, positive staining was obtained using antibodies directed against desmin (A), HNF-3β (B), β-III-tubulin (C) and nestin (D). Areas of spontaneously contracting cells, resembling cardiomyocytes, were also observed (not shown). Taken together, these results indicate that hESC of LOT AL002 are capable of differentiating *in vitro* to cells representing the three germ layers.

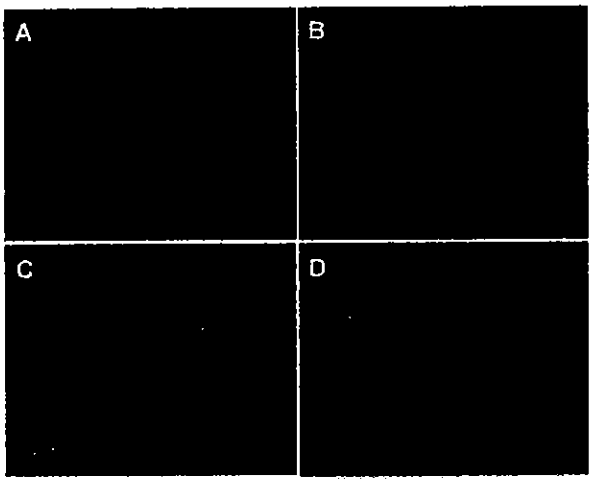

Figure 9. In vitro differentiation of hESC of LOT AL002 in passage 16. (A) desmin, (B) HNF-3β , (C) β-III-tubulin, (D) nestin.

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Pluripotency in vivo

Undifferentiated hESC were surgically placed under the kidney capsule of severe combined immuno-deficient (SCID) mice. The mice were sacrificed after 8 weeks and tumors were dissected and fixed in PFA. Histological evaluation of hematoxylin-eosin stained paraffin sections (Figure 10) demonstrated the presence of tissues derived from endo- (A), meso- (B), and ectoderm (C).

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the policy regarding the use and care of laboratory animals. All research involving animals took place at Laboratory for Experimental Biomedicine which is a specifically pathogen free, full barrier, animal facility at Göteborg University. Göteborg University has PHS Approved Animal Welfare Assurance number A5443-01.

Figure 10. In vivo differentiation of hESC of LOT AL002 in passage 19. (A) Endoderm (secretory epithelium), (B) Mesoderm (cartilage and bone), (C) Ectoderm (neuroectoderm).

Mycoplasma

The presence of mycoplasma in the hESC cultures of LOT AL002 was tested using PCR and mycoplasma specific primers. The assays were performed at the DNA Laboratory at the Department of Clinical Bacteriology, Sahlgrenska University Hospital, Göteborg, Sweden. No mycoplasma was detected.

Human viruses

hESC of LOT AL002 were tested for the presence of Human Immunodeficiency Virus type 1, Hepatitis B, Hepatitis C, Cytomegalovirus, Herpes Simplex Virus type 1 and 2, and Epstein-Barr Virus at the Department of Clinical Virology, Sahlgrenska Academy at the University of Göteborg, Sweden The presence of Human Papilloma Virus was analyzed at the Medical Microbiology Laboratory, University of Lund, Malmö, Sweden. Human Immunodeficiency Virus type 2 was analysed at SMI, Solna, Sweden

None of these viruses were detected.

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Human embryonic stem cell line SA002.5, LOT BE002.5

Background

For the purpose of *in vitro* fertilization (IVF) treatment of patients suffering from involuntary childlessness, human embryos are created at the IVF clinics using conventional IVF-techniques. Supernumerary embryos may, after written informed consent from the donors, be used for research purposes, such as for derivation of human embryonic stem cells (hESC). The hESC derivation process at Göteborg University and Cellartis follows all applicable laws in Sweden and is approved by the Local Research Ethics Committees at Göteborg University and Uppsala University.

Donor confidentiality

In order to protect the privacy and the confidentiality of the donors, all identifiers associated with the embryo donors have been removed. Thus, no information about the donors is accessible. Notably, the donation did not result in any financial gain for the donors.

Summary of characteristics of LOT BE002.5

Parameter	Passage #	Result
Embryo source	--	Frozen, surplus from IVF
hESC line derived	--	May 21, 2001
Procedure for isolation of ICM cells	--	Spontaneously hatched
Subcloning	p155	November 20, 2003
LOT preparation	p155+22	>100 vials
Thawing recovery rate	p155+22-p155+23	100 %
SSEA-1	p155+40	Negative
SSEA-3	p155+40	Positive
SSEA-4	p155+40	Positive
TRA-1-60	p155+40	Positive
TRA-1-81	p155+40	Positive
Oct-4	p155+40	Positive
Alkaline phosphatase	p155+27	Positive
Karyotype	p155+41	*46, XX*
FISH (X, Y, 13, 18 and 21)	p155+31	*Diploid, XX*
Telomerase activity	p155+31	Positive
Pluripotency *in vitro*	p155+29	Endo, ecto, meso
Pluripotency *in vivo*	p155+39	Endo, ecto, meso
Mycoplasma	p155+23	Negative
Human Immunodeficiency Virus type 1 & 2	p155+23	Negative
Hepatitis B	p155+23	Negative
Hepatitis C	p155+23	Negative
Cytomegalovirus	p155+23	Negative
Herpes Simplex Virus type 1 and 2	p155+23	Negative
Epstein-Barr Virus	p155+23	Negative
Human Papilloma Virus	p155+23	Negative

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Details

Derivation of hESC line SA002.5

Establishment and subcloning of hESC lines at Cellartis is performed according to the procedures described in Heins *et al.* (Stem Cells, May 2004) and in Patent application "A method for the establishment of a pluripotent human blastocyst-derived stem cell line" (PCT no. PCT/EP02/14895, Publication no. WO03/055992). For routine expansion, the hESC are cultured on top of a mouse embryonic feeder (MEF) layer using VitroHES™ medium provided by Vitrolife AB (Göteborg, Sweden).

Morphology

At the time of vitrification >100 vials were prepared from the hESC line SA002.5 in passage 155+22. Typical morphology of the hESC colonies, just prior to vitrification, is shown in Figure 2. After thawing and seeding of vitrified cells (*i.e.* LOT BE002.5), viable colonies proliferated and displayed the morphology that characterizes undifferentiated hESC (Figure 3). Subsequently, these cells were propagated and passaged according to standard procedures and representative illustrations of the hESC colonies in passage 155+27, 155+36, 155+42 and 155+56 are shown in Figure 4.

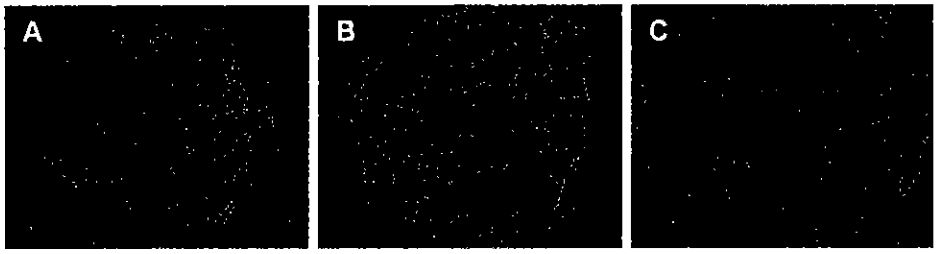

Figure 2 (A)-(C). Typical morphology of hESC line SA002.5 cultured on MEF in passage 155+22 just prior to vitrification.

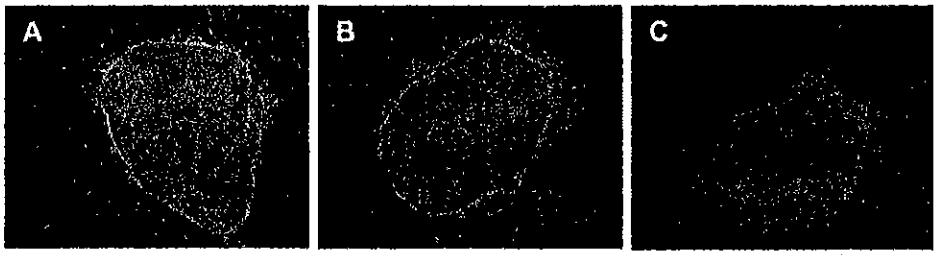

Figure 3 (A)-(C). Typical morphology of hESC cultured on MEF in passage 155+23 after thawing of vitrified cells (LOT BE002.5).

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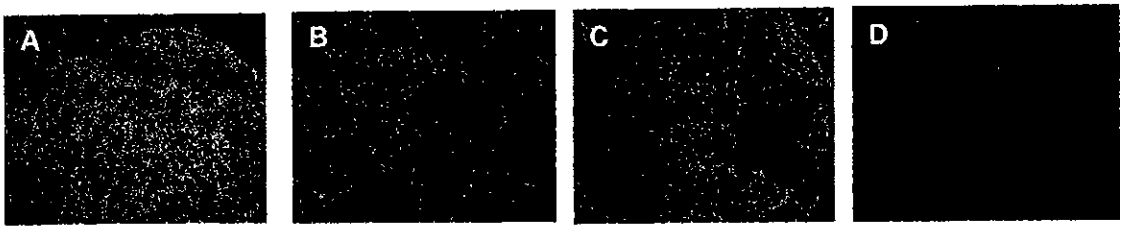

Figure 4. Typical morphology of hESC of LOT BE002.5 cultured on MEF in passage 155+27 (A), passage 155+36 (B), passage 155+42 (C), and passage 155+56 (D).

Thawing recovery rate

The viability of hESC LOT BE002.5 was determined by measuring the thawing recovery rate. Briefly, out of the >100 frozen vials of LOT BE002.5, ten vials were sampled, thawed, and seeded in ten separate dishes containing MEF and VitroHES™ medium. The number of hESC clusters that were seeded, attached, proliferated, and displayed appropriate morphology was determined for each dish. The results are presented in Figure 5 and show that all ten vials (100%) gave rise to viable hESC colonies. These cells were subsequently passaged according to standard procedures and used for the characterization presented in this document.

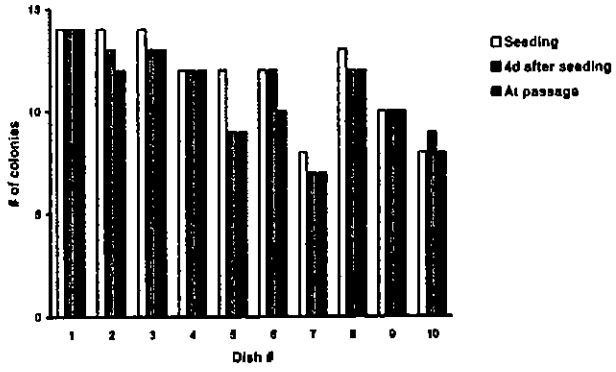

Figure 5. Thawing recovery rate of LOT BE002.5. Thawed hESC were seeded and the number of hESC clumps from each vial was determined (open bars) and subsequently the number of viable colonies was determined four days after seeding (grey bars) and at the time of passage (black bars).

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Immunohistochemical staining of undifferentiated hESC

Undifferentiated hESC colonies of LOT BE002.5 were fixed in PFA and subsequently permeabilized using Triton X-100. After consecutive washing and blocking steps, the cells were incubated with the primary antibody (as indicated in the figure legend). Conjugated secondary antibodies were subsequently used for detection. The nuclei were visualized by DAPI staining. The activity of alkaline phosphatase (ALP) was determined using a commercial available kit following the instructions indicated by the manufacturer (Sigma Diagnostics, Stockholm, Sweden). The passage number at which each analysis was performed is indicated within brackets in the figure legend. The results show that hESC of LOT BE002.5 are negative for SSEA-1 (B) and positive for SSEA-3 (C), SSEA-4 (D), TRA-1-60 (E), TRA-1-81 (F), Oct-4 (G), and ALP (H).

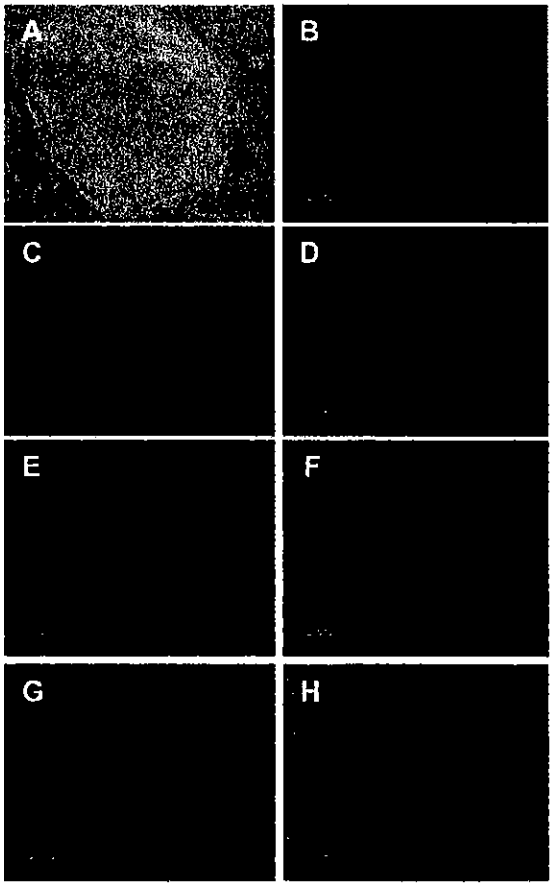

Figure 6 (right). (A) hESC colony [p155+41], (B) SSEA-1 [p155+40], (C) SSEA-3 [p155+40], (D) SSEA-4 [p155+40], (E) TRA-1-60 [p155+40], (F) TRA-1-81 [p155+40], (G) Oct-4 [p155+40], (H) ALP [p155+27]

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Karyotyping

The cells were washed with cell culture medium without serum or serum replacement and then incubated in the presence of Calyculin A. The cells were collected by centrifugation and fixed using ethanol and glacial acetic acid. The chromosomes were visualized using G-banding with a trypsin-Giemsa staining or DAPI (Figure 7).

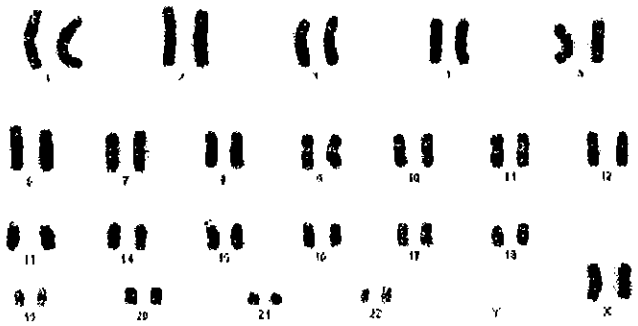

Figure 7. Karyotype of LOT BE002.5 in passage 155+41

FISH

A commercially available kit containing probes for chromosome 13, 18, 21 and the sex chromosomes (X and Y) was used following the instructions from the manufacturer (Vysis. Inc, Downers Grove, IL, USA), with minor modifications. The slides were analyzed in an invert microscope equipped with appropriate filters and software (CytoVision, Applied Imaging, Santa Clara, CA, USA). As illustrated in figure 8, the cells were XX, diploid for chromosomes 13, 18 and 21.

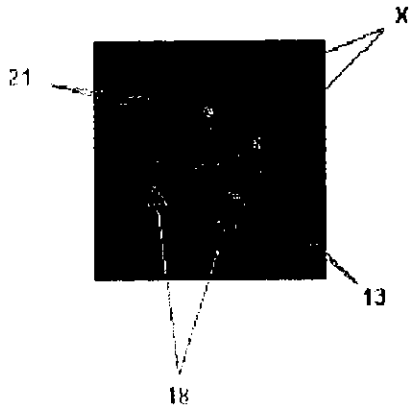

Figure 8. FISH analysis of hESC of LOT BE002.5 in passage 155+31.

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Telomerase activity

For analyzing the telomerase activity a *Telo* TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Basel, Switzerland) was employed according to the manufacturer's instructions. The assay uses the internal activity of telomerase, amplifying the product by PCR and detecting it with an enzyme linked immunosorbent assay (ELISA). hESC of LOT BE002.5 were analyzed in passage 155+31 and displayed high telomerase activity.

Pluripotency in vitro

Undifferentiated hESC colonies were transferred to suspension cultures, using Stem Cell Cutting Tool (Swemed Lab, Göteborg, Sweden), to generate embryoid bodies (EB). Subsequently, these EB were plated in tissue culture plates. Cells that spontaneously differentiated were subjected to immunohistochemical evaluation. As illustrated in figure 9, positive staining was obtained using antibodies directed against Alpha smooth muscle actin (ASMA) (A), HNF-3β (B) and β-III-tubulin (C). Areas of spontaneously contracting cells, resembling cardiomyocytes, were also observed (not shown). Taken together, these results indicate that hESC of LOT BE002.5 are capable of differentiating *in vitro* to cells representing the three germ layers.

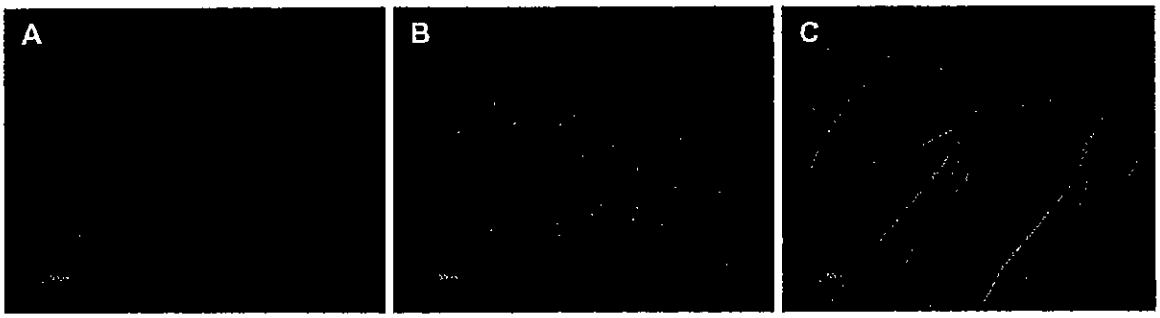

Figure 9. In vitro differentiation of hESC of LOT BE002.5 in passage 155+p29. (A) ASMA, (B) HNF-3β , (C) β-III-tubulin

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Pluripotency in vivo

Undifferentiated hESC were surgically placed under the kidney capsule of severe combined immuno-deficient (SCID) mice. The mice were sacrificed after 8 weeks and tumors were dissected and fixed in PFA. Histological evaluation of hematoxylin-eosin stained paraffin sections (Figure 10) demonstrated the presence of tissues derived from endo- (A), meso- (B), and ectoderm (C).

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the policy regarding the use and care of laboratory animals. All research involving animals took place at Laboratory for Experimental Biomedicine which is a specifically pathogen free, full barrier, animal facility at Göteborg University. Göteborg University has PHS Approved Animal Welfare Assurance number A5443-01.

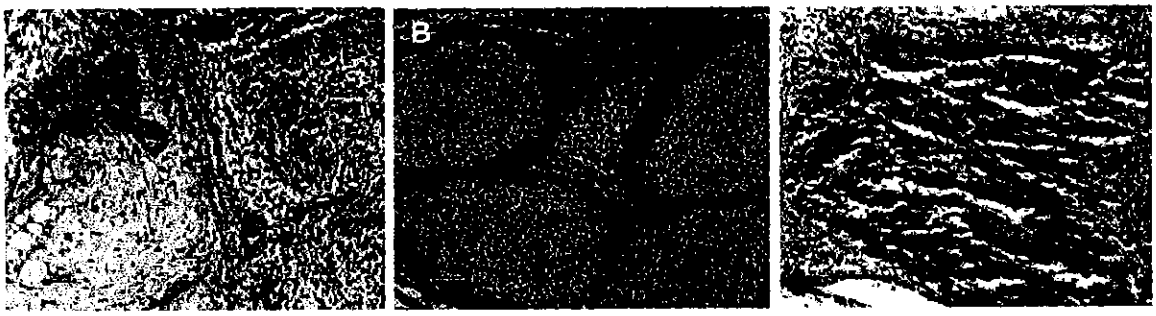

Figure 10. In vivo differentiation of hESC of LOT BE002.5 in passage 155+39 (A) Endoderm (secretory epithelium), (B) Mesoderm (cartilage), (C) Ectoderm (neuroectoderm).

Mycoplasma

The presence of mycoplasma in the hESC cultures of LOT BE002.5 was tested using PCR and mycoplasma specific primers. The assays were performed at the DNA Laboratory at the Department of Clinical Bacteriology, Sahlgrenska University Hospital/SU, Göteborg. No mycoplasma was detected.

Human viruses

hESC of LOT BE002.5 were tested for the presence of Human Immunodeficiency Virus type 1 and 2, Hepatitis B, Hepatitis C, Cytomegalovirus, Herpes Simplex Virus type 1 and 2, and Epstein-Barr Virus at the Department of Clinical Virology, Sahlgrenska Academy at Göteborg University/SU, Göteborg. The presence of Human Papilloma Virus was analyzed at Medical Microbiology, Lund University, Malmö. None of these viruses were detected.

STEM CELLS®
Original Article

Derivation, Characterization, and Differentiation of Human Embryonic Stem Cells

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ABSTRACT

The derivation of human embryonic stem (hES) cells establishes a new avenue to approach many issues in human biology and medicine for the first time. To meet the increased demand for characterized hES cell lines, we present the derivation and characterization of six hES cell lines. In addition to the previously described immunosurgery procedure, we were able to propagate the inner cell mass and establish hES cell lines from pronase-treated and hatched blastocysts. The cell lines were extensively characterized by expression analysis of markers characteristic for undifferentiated and differentiated hES cells, karyotyping, telomerase activity measurement, and pluripotency assays in vitro and in vivo. Whereas three of the cell lines expressed all the characteristics of undifferentiated pluripotent hES cells, one cell line carried a chromosome 13 trisomy while maintaining an undifferentiated pluripotent state, and two cell lines, one of which carried a triploid karyotype, exhibited limited pluripotency in vivo. Furthermore, we clonally derived one cell line, which could be propagated in an undifferentiated pluripotent state. *Stem Cells 2004;22:367-376*

INTRODUCTION

The inner cell mass (ICM) of the preimplantation blastocyst contains a core of cells, termed the epiblast, that have the potential to generate somatic and germ cells of the embryo. It has previously been demonstrated that human embryonic stem (hES) cell lines, exhibiting a stable developmental potential to form derivatives of the three germ layers after prolonged culture in vitro, can be generated by the isolation and culturing of the human ICM [1, 2]. So far, the available knowledge of conditions for deriving, characterizing, and culturing undifferentiated hES cells is largely based on a relatively few successfully isolated cell lines. Furthermore,

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the underlying mechanisms that control the developmental decisions of hES cells in culture remain essentially unknown. It has been suggested that the poor success rates in developing ES cells in species other than mice is either due to fundamental biological differences between the species or simply due to technical factors associated with the derivation and culture conditions [3, 4]. For example, unless spontaneous differentiation of hES cells is prevented, the cells become gradually restricted and lose the characteristics of ES cells. Frequent passaging and optimization of the quality of the culture conditions and feeder cells may overcome this problem. However, many problems remain with regard to the optimal maintenance and expansion of hES cells.

Due to the limited number of available hES cell lines, there is an urgent need for the generation and characterization of more cell lines, as each line may have its own characteristics and advantages for different applications. Furthermore, the availability of more hES cell lines for comparison will aid in defining criteria for bona fide hES cells and the establishment of appropriate and robust methods for maintenance and expansion of hES cells.

Here, we describe the successful establishment of hES cell lines from the ICM by immunosurgery, from spontaneously hatched blastocysts, and from blastocysts after pronase-mediated removal of the zona pellucida. Three of the hES cell lines have been maintained in culture for more than 1 year, during which time high levels of telomerase activity, stable karyotype, and expression of markers characteristic for undifferentiated hES cells were maintained. The cells could be cryopreserved by vitrification without any effect on their ability to re-establish pluripotent hES cell colonies. The pluripotent qualities of these cell lines were demonstrated in several ways. Most importantly, the cells were able to differentiate into cell types originating from each of the three embryonic germ layers (endoderm, mesoderm, and ectoderm) in vitro as well as in vivo. In addition, we subcloned one of our cell lines and showed that it could be propagated in an undifferentiated state while maintaining its pluripotency both in vitro and in vivo, as was previously shown for other hES cell lines [5].

Materials and Methods

Establishment and Culture of Human Embryonic Stem Cell Lines

Surplus human embryos from clinical in vitro fertilization (IVF) treatment were donated after informed consent and approval of the local ethics committees at Göteborg University and Uppsala University. Donated embryos were cultured to blastocysts until the age of 6-7 days as previously described [6, 7]. Blastocysts were graded (grade shown within brackets) according to *Dokras et al.* [8] and randomly selected for either pronase treatment (lines Fertilitetscentrum [FC]018 [B], Akademiska sjukhuset [AS]034 [A], and AS038 [B]) or pronase treatment followed by immunosurgery (lines Sahlgrenska [SA]121 [A], SA181 [B]) (see below). Spontaneously hatched blastocysts (line SA002 [B]) were placed directly in VitroHES supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (hrbFGF) (GIBCO/Invitrogen; Paisley, UK; http://www.invitrogen.com), and 125 μg/ml hyaluronic acid (HA) (Ophthalin, CIBA Vision Nordic AB; Askim, Sweden; http://www.cibavision.no) on a layer of mitotically inactivated early passage mouse embryonic fibroblasts (MEFs) (F1 hybrid CD1xC57BL/6, Charles River Laboratories; Sulzfeld, Germany; http://www.criver.com). VitroHES was composed as previously described [5] and manufactured by Vitrolife AB (Kungs-backa, Sweden; http://www.vitrolife.com) where it was subjected to physical and functional tests as part of a quality control program to increase the final medium quality. The MEF cells were derived and cultured as previously described [9]. Briefly, the mitotic activity of the MEFs was abolished by an incubation with 10 μg/ml mitomycin C (Sigma-Aldrich Sweden AB; Stockholm, Sweden; http://www.sigmaaldrich.com) for 3 hours at 37°C, after which the cells were seeded at a density of 130,000 cells/ml in IVF cell culture dishes (Falcon 3653, Becton Dickinson; Franklin Lakes, NJ; http://www.bd.com) in MEF medium (Dulbecco's-modified Eagle's medium [D-MEM]) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, and 1× Glutamax (Invitrogen, Sigma-Aldrich). Prior to the addition of treated blastocysts or hES cells, the MEF medium was changed to VitroHES.

Blastocysts with intact zona were treated in pronase for 1-3 minutes (Sigma-Aldrich:10 U/ml in ICM-2 [Vitrolife AB] 1-3 minutes in three subsequent drops), washed three times in ICM-2 and plated on MEFs in hES medium supplemented with 125 mg/ml HA and 4 ng/ml hrbFGF. ICM-2 is a blastocyst culture medium containing recombinant human albumin and HA. Pronase-treated zona-free blastocysts selected for immunosurgery were washed twice in antihuman serum antibody (Sigma-Aldrich, 1:5 in ICM-2). After the second wash the blastocysts were placed in a new drop of the antibody and incubated for 30 minutes. The blastocysts were then extensively washed three times in ICM-2 medium followed by three washes in guinea-pig complement serum (Invitrogen, 1:5 in ICM-2). The blastocysts were then incubated for 10 minutes in guinea-pig complement serum and followed by three washes in ICM-2 and placed on MEFs in VitroHES supplemented with 125 μg/ml HA and 4 ng/ml hrbFGF.

ICM outgrowths were passaged to plates with fresh medium and MEF cells by mechanical dissection using Stem

Cell Tool™ (Swemed Lab International AB; Billdal, Sweden). Established hES cell lines were routinely passaged every 4-5 days. The hES cell colonies were mechanically cut into pieces, 200 × 200 μm, and removed from the culture dish and transferred to a new culture dish with fresh MEF cells and VitroHES supplemented with 4 ng/ml hrbFGF.

Telomerase Activity

It has been demonstrated that high telomerase activity in ES cells correlates with their ability to divide indefinitely in culture [10]. Degradation of telomeric sequences or end-to-end fusion of chromosomes can lead to genomic instability [11]. To analyze telomerase activity in hES cells a polymerase chain reaction-based enzyme-linked immunosorbent assay was used (Roche Diagnostics GmbH; Mannheim, Germany; http://www.roche.com). The cells were harvested and lysed according to the manufacturer's instructions.

Karyotyping and FISH

hES cells designated for karyotyping were cultured in hES medium supplemented with 0.1 μg/ml colcemid (Invitrogen) for 1-3 hours. The cells were subsequently trypsinized, fixed, and mounted on glass slides. The chromosomes were visualized by using modified Wright's staining. For fluorescence in situ hybridization (FISH) analysis, a commercially available kit containing probes for chromosome 13, 18, and 21 and the sex chromosomes (X and Y) was used (MultiVysion™ PB Multicolour Probe Panel; Vysis, Inc.; Downers Grove, IL; http://www.vysis.com) according to the manufacturer's instructions. For each cell line at least 200 nuclei were analyzed. The slides were analyzed in a fluorescence microscope equipped with appropriate filters and software (CytoVision; Applied Imaging; Santa Clara, CA; http://www.appliedimagingcorp.com).

In Vitro Differentiation

The cells were kept on MEFs without passaging up to 14 days. Medium (hES medium without hrbFGF) was changed every second day. Alternatively, incubating clumps of hES cells in VitroHES without hrbFGF in suspension culture for 4-9 days generated both simple and cystic embryoid bodies (EBs).

Xenografting of hES Cells

Severe combined immunodeficient (SCID) mice, [12] (C.B-17/IcrCrl-scidBR; Charles River Laboratories) were used as animal hosts for the xenografted hES cells. Four- to five-week-old animals were anesthetized with intraperitoneal injections of ketamine hydrochloride (Ketalar; Warner Lambert Nordic AB; Solna, Sweden; http://www.warner-lambert.com; 75 μg/g mouse) and medetomidine hydrochloride (Domitor; Orion Pharma Corporation; Espoo, Finland; http://www.orionpharma.com; 1 μg/g mouse). hES cell colonies were mechanically cut into 200 × 200-μm pieces, washed once in phosphate-buffered saline (PBS) containing 4 mg/ml human serum albumin and penicillin (Cryo-PBS; Vitrolife AB), and 20 cell clusters were injected under the kidney capsule or in the testicular lumen using a 200-μm lumen glass transfer pipette (Swemed Lab International AB). The number of cells transferred was approximately 20,000 to 40,000 per organ. Control animals were either injected with Cryo-PBS or grafted with primary brain cells from a littermate. The mice were resuscitated with intraperitoneal injections of atipamezol (Antisedan; Orion Pharma; 1 μg/g mouse), and kept on a heated pad until consciousness. Palpable tumors started to appear 3 weeks after transplantation. The tumors were allowed to develop for 8 weeks before the animals were sacrificed by cervical dislocation. All animals appeared healthy during the 8-week period, and no animal died due to illness. The tumors were excised and immediately fixed in 4% paraformaldehyde, incubated for 24 hours, washed or stored in 70% ethanol, and processed in a Tissue-Tek paraffin infiltrator (Sakura Fine Technical; Tokyo, Japan; http://www.sakuraus.com), and paraffin-embedded. The tumors were subsequently sectioned in 6-8 μm sections with a Microm HM 360 (Microm GmbH; Walldorf, Germany). Samples were stained for morphology using hematoxylin and eosin with a Sakura DRS-601 stainer. To determine the origin (human, mouse) and tissue type within teratomas, we combined histopathological and marker expression analysis. To evaluate whether the tissues were of human origin we used several human-specific antibodies, e.g., anti-E-cadherin and anti-human nuclei (see below). To confirm the presence of tissues derived from all three germ layers, we focused on tissues that can be easily distinguished by histopathological methods, e.g., neuroectoderm, cartilage, kidney tubuli, and gut-like epithelium. To strengthen these conclusions, we also used antibodies against markers characteristic for derivatives of the germ layers, e.g., α-smooth muscle actin, desmin, nestin, β-III-tubulin, α-fetoprotein, and HNF3β.

Subcloning

The cells were washed inside the wells twice with cloning medium, 150 μl, 500 μl, and 1,000 μl for the 96-, 48-, 24-well plates, respectively. The inner part of the colonies was cut with a 300-μm Stem Cell Tool™ and subsequently incubated with 0.5 mM EDTA for 20 minutes at 37°C. The cells were triturated carefully with a pipette and diluted either in knockout (KO)-DMEM medium (GIBCO) supplemented with 15% concentrated conditioned medium, 3.5 mM glucose, 1 mM Glutamax (invitrogen-sigma-albumin), 1% NEAA (GIBCO),

and 4 ng/ml bFGF, KO-DMEM medium supplemented with 15% FCS, 3.5 mM glucose, 1 mM Glutamax, 1% NEAA, and 4 ng/ml bFGF, or KO-DMEM medium supplemented with 20% serum replacement (GIBCO) SR, 3.5 mM glucose, 1 mM Glutamax, 1% NEAA, and 4 ng/ml bFGF. Single cells were picked and put into individual wells with MEF-coated plates. To confirm the colony-forming ability of the cells, positive controls were performed (e.g., 10, 100, 1,000 cells/ml or smaller clusters) as well as negative controls (wells without dissociated hES cells). Subclones were preferentially obtained in 15% concentrated conditioned medium of hES cells grown in presence of FCS.

Histochemical Staining for Alkaline Phosphatase

Histological staining for alkaline phosphatase was carried out using a commercially available kit (Sigma-Aldrich) following the manufacturer's instructions.

Immunocytochemistry

The cells were fixed in 4 % paraformaldehyde for 15 minutes at room temperature, washed in PBS, and exposed to the primary antibodies overnight at 4°C. As secondary antibodies, we used fluorescein isothiocyanate (FITC)- and Cy-3-conjugated antibodies (1:50, Southern Biotech; Birmingham, AL; http://www.southernbiotech.com). The monoclonal antibodies (mAb) directed against SSEA-1, SSEA-3, and SSEA-4 (Developmental Studies Hybridoma Bank, The University of Iowa; Iowa City, IA) were used at 1:200, whereas the TRA-1-60 and TRA-1-81 mAbs (Santa Cruz Biotechnology; Santa Cruz, CA; http://www.southernbiotech.com) were used at 1:500. Neuroectodermal precursor cells and neurons were detected by a nestin mAb (BD Biosciences; Stockholm, Sweden; 1:100), and β-tubulin-III mAb (Sigma-Aldrich; 1:100), respectively. Endodermal cells were recognized by a mAb against Cdx2 (gut endoderm, visceral endoderm) (BioGenex, Nordic BioSite; Täby, Sweden; 1:200; http://www.innogenex.com), and polyclonal antibodies against α-1-fetoprotein ([AFP] Sigma-Aldrich; 1:2000) and HNF3β (Santa Cruz Biotechnology; 1:500). Mesodermal cells were detected by a desmin antibody (Chemicon; Temecula, CA; http://www.chemicon.com; 1:200). hES cell-derived cells were detected by mAbs against human nuclei (Chemicon; 1:100) and human E-cadherin (Zymed Laboratories; South San Francisco, CA; http://www.zymed.com; 1:500). Some cultures were double stained with DAPI (4′-6′Diamidino-2-phenylindole, final concentration 0.1 μg/ml, Sigma-Aldrich) for 5 minutes.

Results

Methods for Deriving hES Cell Lines

The most commonly used method for deriving hES cell lines is by immunosurgical isolation of the ICM from the human blastocyst [1, 2, 13]. Here, we show that hES cell lines can be established from pronase-treated and hatched blastocysts as well (Fig. 1). Due to the limited number of embryos used in this study, we can, however, not conclude anything about the relative efficiencies of the different methods. Whereas cell line SA002 was derived from a spontaneously hatched blastocyst, cell lines FC018, AS034, and AS038 were established from pronase-treated blastocysts (Table 1). Immunosurgery was used for establishing cell lines SA121 and SA181. One to two weeks after plating, the expanded ICM was transferred to a fresh MEF-coated IVF-cell culture dish by mechanical dissection. Successful propagation of the ICM was associated with the appearance of ES-like cells in the outgrowth, whereas differentiated cells, presumably representing primitive endoderm and trophectoderm, either died or disappeared upon repeated passaging (Fig. 1).

Characterization of hES Cell Lines

To analyze the long-term pluripotency and replicative immortality of the six newly established hES cell lines

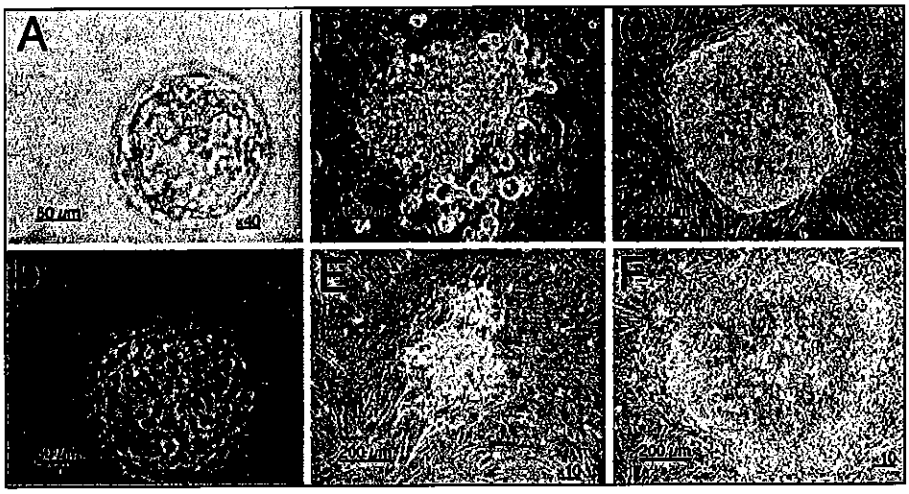

Figure 1. Human ES cell derivation from pronase-treated and hatched blastocysts. A) Blastocyst before pronase treatment. B) Outgrowth of pronase-treated blastocyst shown in (A) 6 days after pronase treatment. C) hES colony (passage 6) derived from the ICM in (B). D) Spontaneously hatched blastocyst. E) Outgrowth of the blastocyst shown in (D) 5 days after plating. F) hES colony (passage 3) derived from the ICM in (E).

Table 1. Summary of hES cell characterization

hES line	Isolation-method	SSEA-1	SSEA-3	SSEA-4	TRA-1-60	TRA-1-81	ALP	Oct-4
*SA002	Spontaneous hatching	–	+	+	+	+	+	+
FC018	Pronase	±	±	±	+	+	+	+
AS034	Pronase	–	+	+	+	+	+	+
AS038	Pronase	±	±	±	+	+	+	+
SA121	Immunosurgery	–	+	+	+	+	+	+
SA181	Immunosurgery	–	+	+	+	+	+	+
AS034.1	Subcloning	–	+	+	+	+	+	+

hES line	Karyotype	Telomerase	FISH	In vitro differentiation	Teratoma
*SA002	47 XX	++	+13 XX	Three germ layers	Three germ layers
FC018	69 XXY	++	Trip. XXY	Three germ layers	Fluid-filled cysts
AS034	46 XY	++	2n XY	Three germ layers	Three germ layers
AS038	46 XY	+	2n XY	Three germ layers	Fluid-filled cysts
SA121	46 XY	++	2n XY	Three germ layers	Three germ layers
SA181	46 XY	++	2n XY	Three germ layers	Three germ layers
AS034.1	46 XY	n.d.	2n XY	Three germ layers	Three germ layers

*NIH-eligible cell line

n. d. = not determined

SA002, FC018, AS034, AS038, SA121, SA181, and subclone AS034.1 (see below), we used previously defined criteria for in vitro and in vivo characterization of hES cells by examining the morphology, marker expression, telomerase activity, karyotype, and pluripotency in vitro and in vivo.

In the presence of mouse feeders and human recombinant bFGF, all six hES cell lines gave rise to large compact multicellular colonies of cells with the characteristic hES cell morphology, i.e., a high ratio of nucleus to cytoplasm and prominent nucleoli. Some of these lines have been passaged more than 120 times. Initially, the different cell lines could not be discriminated morphologically from each other except for cell line AS038, which never developed a clear distinguishable border towards the mouse feeders (data not shown). However, with time the morphology of AS038 became indistinguishable from the other cell lines.

Karyotype analyses carried out at different passages (from passage three to passage 76) indicated a normal stable karyotype in four of the cell lines (Fig. 2A, Table 1). In two of the cell lines chromosomal aberrations were apparent, trisomy 13 in cell line SA002 (Fig. 2B), and triploid karyotype in FC018 (Table 1).

Similar to undifferentiated pluripotent cultures of human germ cells [14] and previously established hES cell lines, all our cell lines possessed high levels of alkaline phosphatase (AP) activity (Fig. 3B, Table 1). The overall percentage of visible AP-positive cells within a colony varied from 60% to 90% in all cell lines. The hES cells lines

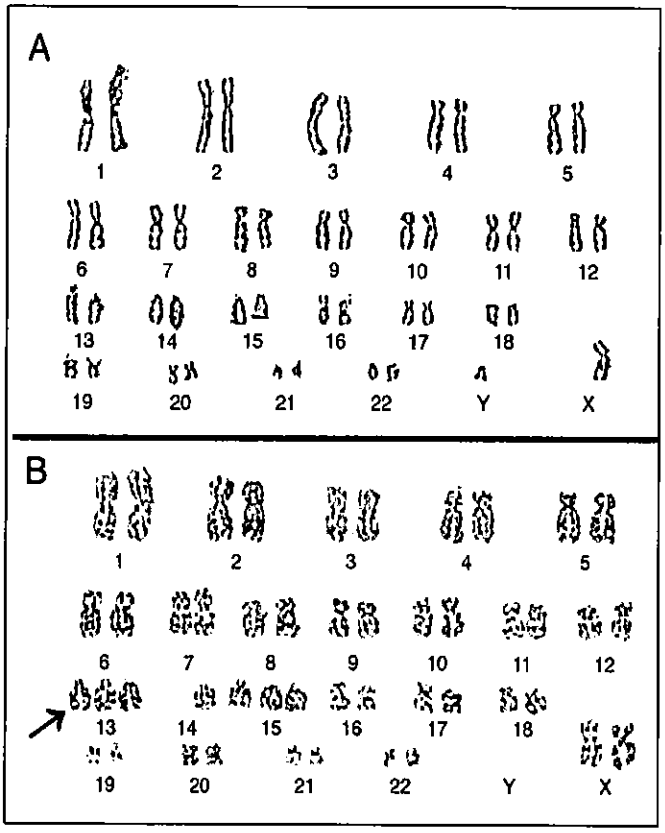

Figure 2. Karyotype of hES lines (see also Table 1). *A) Normal 46XY karyotype from cell line SA181. B) XX karyotype from line SA002 with a trisomy 13. The arrow in (B) points to the trisomic chromosome 13.*

were further characterized by expression analysis of five cell surface markers: SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, and the intermediate filament protein nestin [1, 14-17]. Figure 3 depicts examples of the expression of these markers in undifferentiated colonies from cell line SA002. The results of the expression analysis are summarized in Table 1. Whereas all cell lines expressed SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, they were, with a few exceptions (see below), negative for nestin and SSEA-1.

Oct-4 is a POU-domain transcription factor that is essential for establishment of ES cells from the ICM [18]. Similar to previous reports [19, 20], we found that undifferentiated hES cells expressed Oct-4 (Fig. 3H, Table 1), whereas Oct-4 was downregulated concomitant with differentiation (data not shown).

Nestin is expressed in a variety of stem/precursor cell populations of neuroectodermal and mesodermal origin, and we found it useful for detecting differentiated hES cells that could not be recognized by morphological criteria. Generally, nestin-positive colonies often appeared during suboptimal growth conditions and seemed to result in irreversible commitment. Interestingly, whereas TRA-1-60 and TRA-1-81 were initially expressed normally in cell lines FC018 (passage 25) and AS038 (passage 41) (Fig. 4C and 4D, Table 1), a patchy expression pattern of SSEA-3 and SSEA-4 was observed (Fig. 4F, Table 1). Moreover, additional signs of cell differentiation were the appearance of

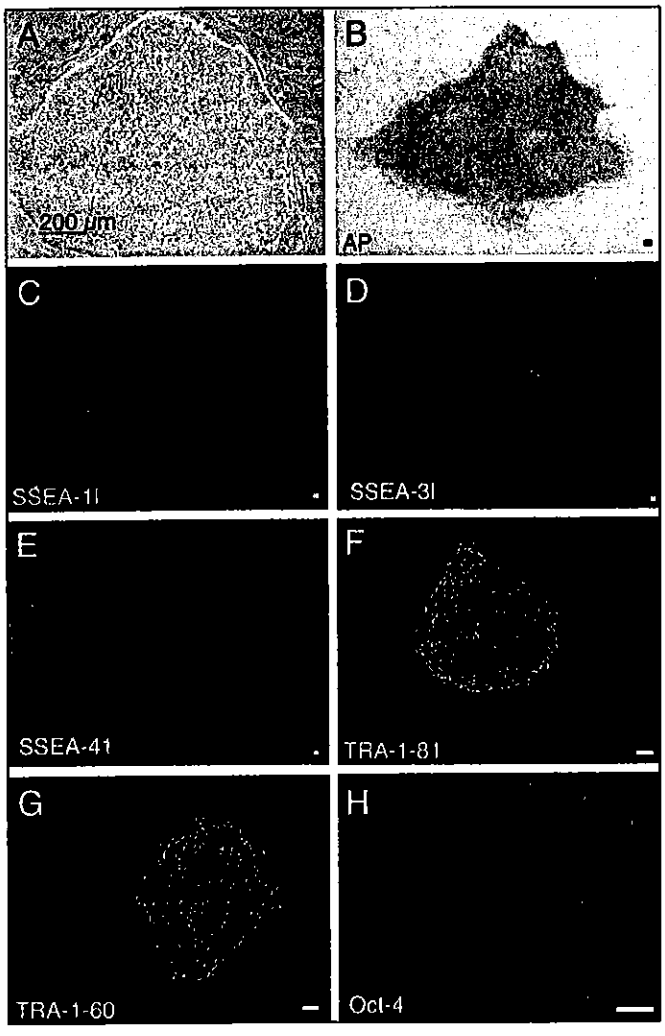

Figure 3. Immunohistochemical marker expression analysis of cell line SA002 (passage 21). The colonies were analyzed 5 days after passaging. A) Morphology, (B) Alkaline Phosphatase (AP), (C) SSEA-1, (D) SSEA-3, (E) SSEA-4, (F) TRA-1-81, (G) TRA-1-60, and (H) Oct-4. Scale bar: 25 µm.

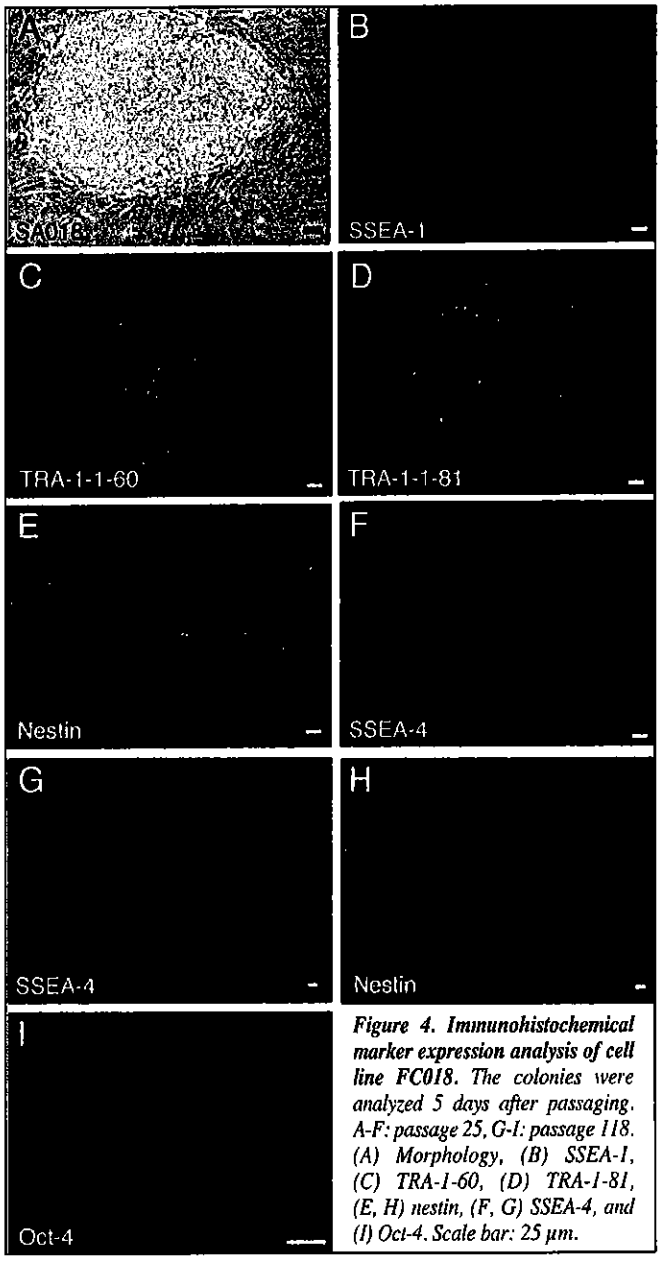

Figure 4. Immunohistochemical marker expression analysis of cell line FC018. The colonies were analyzed 5 days after passaging. A-F: passage 25, G-I: passage 118. (A) Morphology, (B) SSEA-1, (C) TRA-1-60, (D) TRA-1-81, (E, H) nestin, (F, G) SSEA-4, and (I) Oct-4. Scale bar: 25 µm.

SSEA-1- and nestin-expressing cells (Fig. 4B and 4E, Table 1). However, with time both line FC018 (passage 118) and AS038 (passage 72) expressed SSEA-4 and Oct-4 uniformly within the colonies (Fig. 4G and 4I), whereas nestin was no longer expressed (Fig. 4H).

Finally, all six hES cell lines expressed high levels of telomerase activity that were maintained even after more than 50 passages (Table 1).

In summary, three of the six characterized hES cell lines, AS034, SA121, and SA181, exhibited the morphology, genotype, telomerase activity, and marker expression characteristics for previously reported pluripotent stem cell lines with a normal karyotype [1, 2, 13].

Analysis of In Vitro Pluripotency of hES Cell Lines

Similar to mouse ES cells, hES cells spontaneously form three-dimensional aggregates of differentiated cells known as EBs when grown in suspension. Upon continued in vitro culture of EBs, a variety of ectodermal, endodermal, and mesodermal germ layer derivatives, such as hematopoietic, endothelial, cardiac, skeletal muscle, and neuronal cell lineages appear [21]. We could show that all hES cell lines are capable of generating both simple and cystic EBs. Marker expression analysis and morphological examination of plated EBs revealed derivatives of all three germ layers, including areas of beating heart muscle-like cells (data not shown). However, EB formation is not an exclusive pathway for initiating hES cell differentiation. An alternative efficient and timesaving method to induce spontaneous differentiation of hES cells is simply by keeping the colonies on mouse feeders for more than 7 days without passaging. Similar to EB formation, this method gives rise to a variety of cell types derived from all three germ layers. The vast majority of cells within the differentiated colonies expressed neuroectodermal cell markers, such as nestin and β-III-tubulin (Fig. 5A and 5B). These markers were preferentially expressed within typical rosette-like structures during early stages of differentiation (data not shown). Derivatives of mesoderm were confirmed by desmin stainings, and the appearance of synchronously beating cardiomyocyte-like cells (Fig. 5C, data not shown). Endodermal derivatives appeared later during differentiation in the periphery of the colonies and were identified by the expression of AFP, Cdx2, and HNF3β (Fig. 5D-5F). In summary, based on in vitro differentiation, all cell lines displayed the potential to form derivatives of all three embryonic germ layers. Importantly, these characteristics remained the same after repeated freezing-thawing cycles (data not shown).

Since none of the described hES cell lines were clonally derived, it cannot be excluded that multiple precursor or stem cells committed to different lineages may coexist within a population of homogeneously appearing cells. Theoretically, this would imply that, until proven, it cannot be stated that a single hES cell is capable of forming derivatives of all three embryonic germ layers. In our initial efforts to subclone cell line AS034, we obtained one clone, AS034.1. To promote cell survival, we used concentrated conditioned medium from hES cells grown in presence of FCS as cloning medium (**Materials and Methods**). However, the overall yield was low; on average from approximately 10^3 dissociated single cells one colony resulted. Characterization of subclone AS034.1 revealed that it behaved comparably to the other cell lines in terms of the expression of SSEA-4, SSEA-3, TRA-1-60, and TRA-1-81 (Fig. 6). Importantly, SSEA-1 and nestin were not detected in undifferentiated colonies (Fig. 6B, data not shown). Furthermore, the subclone was capable of differentiating into ectodermal, mesodermal, and endodermal cell types both in vitro and in vivo (Table 1).

Analysis of In Vivo Pluripotency of hES Cell Lines

When clusters of hES colonies are xenotransplanted to SCID mice, they form teratomas consisting of cell types

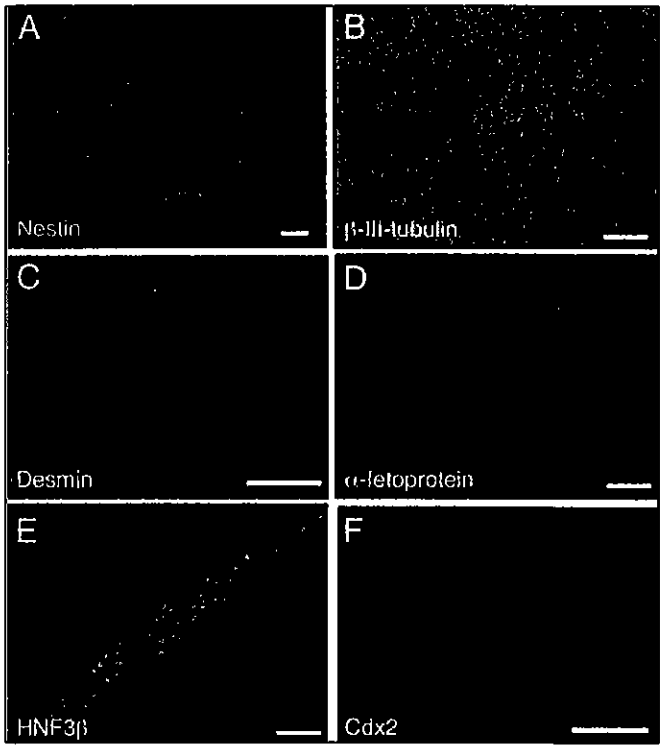

Figure 5. In vitro differentiation of cell line SA002. *The colonies were analyzed after 12 days after passaging. A) Nestin-positive neuronal precursors, B) β-III-tubulin-positive postmitotic neurons, C) desmin-positive mesodermal cells, D, E, F) α-fetoprotein-positive (D), HNF3β-positive (E), and Cdx2-positive (F) endodermal cells. Scale bar: 25 μm.*

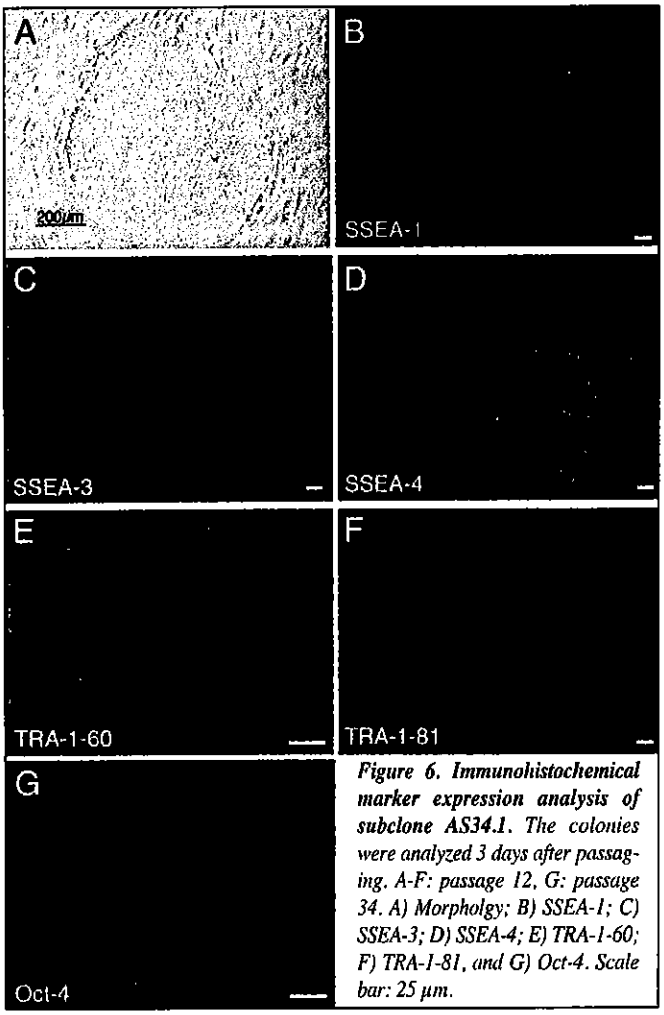

Figure 6. Immunohistochemical marker expression analysis of subclone AS34.1. *The colonies were analyzed 3 days after passaging. A-F: passage 12, G: passage 34. A) Morpholgy; B) SSEA-1; C) SSEA-3; D) SSEA-4; E) TRA-1-60; F) TRA-1-81, and G) Oct-4. Scale bar: 25 µm.*

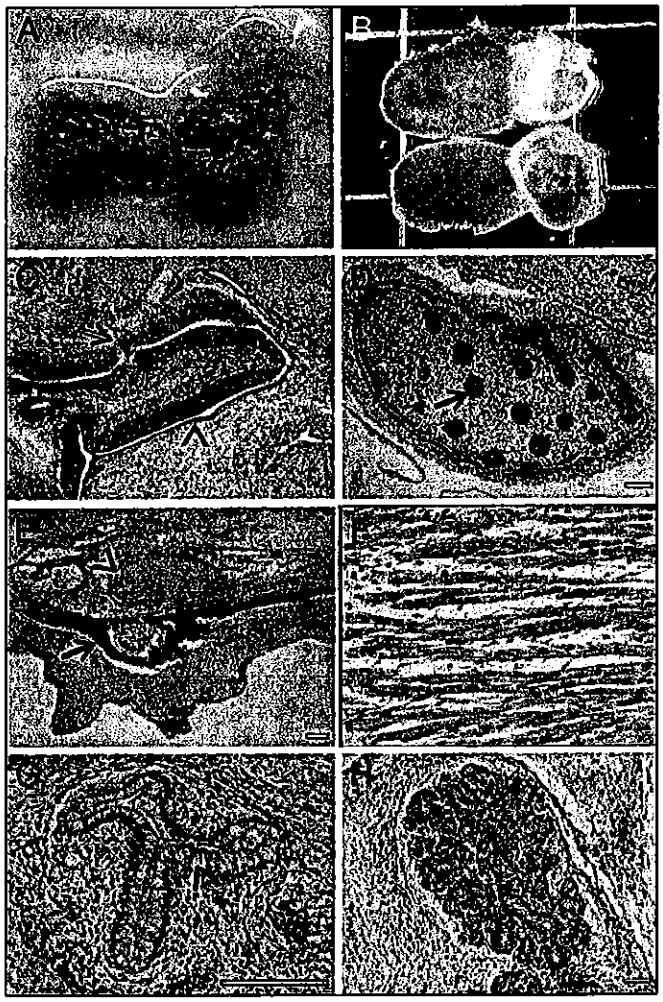

Figure 7. Teratoma analysis. *A, B) Low-power view of a solid tumor from cell line SA121 (A) and a fluid-filled cyst generated from cell line FC018 (B). C, D) Derivatives of the ectoderm. Pigmented epithelium (arrowhead) and neuroepithelium (arrow) are shown in C and hair follicles (arrow) in D. E, F) Derivatives of the mesoderm. Cartilage (arrowhead) and bone (arrow) are shown in E, and skeletal muscle in F. G, H) Derivatives of the endoderm. Gut-like epithelium with mucous-containing cells (arrowhead) and glandular epithelium are shown in G and H, respectively. Scale bars: 100 µm.*

derived from ectoderm, mesoderm, and endoderm. To analyze the in vivo pluripotency of the derived hES cell lines, we transplanted clusters of hES cells under the kidney capsule of SCID mice. Eight weeks after the transplantation the mice were sacrificed and teratomas were analyzed. To distinguish human cells from mouse cells, we used antibodies specific for human-specific nuclear antigen [22] and human E-cadherin. Xenografting of hES cluster resulted in two morphologically distinct structures in the kidneys. From a majority of the cell lines (SA002, AS034, AS034.1, SA121, and SA181) solid teratomas, consisting of highly differentiated cells and tissues derived from all three germ layers, such as gut epithelium; glandular epithelium (endoderm); cartilage, bone, smooth muscle, striated muscle, and kidney glomeruli-like structures (mesoderm); and pigment epithelial cells, neural epithelium, hair follicles, and stratified squamous epithelium (ectoderm) formed (Fig. 7, Table 1, data not shown). However, two of the cell lines (FC018, AS038) consistently formed fluid-filled cyst-like structures composed of hES cell-derived connective tissue and epithelial cells (Fig. 7B, Table 1). Infrequently, these cysts contained small solid teratomas consisting of cell types derived from several germ layers (data not shown). Thus, in summary, whereas all cell lines, including lines with an abnormal karyotype, exhibited pluripotent differentiation qualities in vitro, pluripotency in vivo was only consistently observed in lines SA002, AS034, AS034.1, SA121, and SA181

Discussion

Here we report the derivation and characterization of six new hES cell lines. With a few exceptions the cell lines

behaved like previously reported blastocyst-derived pluripotent stem cell lines. We believe that the phenotypic discordance in vitro and in vivo of some of the characterized cell lines is of value both for evaluating present characterization tools and for their further improvement in order to set up robust criteria for analyzing hES cells.

In addition to the previously reported immunosurgery protocol [1, 23], we report the successful derivation of hES cell lines from a spontaneously hatched blastocyst, and from blastocysts after enzymatic removal of the zona pellucida by pronase. A possible drawback of only removing the zona is overgrowth by the trophectoderm and the possible generation of trophectoderm stem cell lines. However, this should also be taken into account when using immunosurgery since this method does not guarantee complete removal of trophectoderm cells. Nevertheless, due to the morphology of trophoectoderm cells, i.e., flattened and polarized as they mature [24], they can be discriminated from the ICM once plated on MEFs. The fact that all of our cell lines expressed Oct-4, which normally is downregulated during differentiation and in trophectoderm stem cells [18, 19], substantiates our conclusion that the derived pluripotent stem cells were derived from the ICM.

Although four of our cell lines appeared phenotypically similar to previously derived hES cell lines [1, 2, 13], two of the cell lines (FC018 and AS038) were phenotypically different. Instead of generating solid teratomas upon xenografting, they developed into cyst-like structures. In contrast, when differentiated in vitro these lines generated cell types from all three germ layers. Except for their morphology in culture, which was similar to the "normal" hES cell lines, FC018 and AS038 also exhibited aberrant expression patterns of some of the markers characteristic for undifferentiated and differentiated hES cells, respectively. However, with time, the expression of these markers became reminiscent of normal hES cells. Whether the temporal shift in marker expression pattern in these cell lines can be explained by an initial mixed population of cells which went through selection, or whether cells were more prone to spontaneously differentiate initially, is presently unclear.

Our results indicate that adding early differentiation markers, such as nestin, to the list of markers whose expression patterns are analyzed in undifferentiated hES cells improves the detection of early hES cell differentiation. We show that the expression of nestin, a marker for stem/precursor cells of neuroectodermal and mesodermal origin, precede visible morphological signs of differentiation. We also found that the frequent spontaneous commitment to the neuroectodermal pathway of cell lines FC018 and AS038 correlate with loss of developmental potential to form derivatives of the three germ layers in vivo.

By definition, clonal expansion of hES cells is a prerequisite for the strict definition of pluripotent cell lines. Currently, the culture conditions for clonal expansion of hES cells are suboptimal. Unlike mouse ES cells, hES cells die at a high rate when they are dissociated into single cells [5]. Depending on which cell line was used, only 0.1%-1% of plated single cells was able to generate colonies that could be propagated. Among the few clones that survived, the majority were lost due to irreversible differentiation. We found that concentrated conditioned medium from hES cells grown in the presence of FCS promoted cell survival and maintenance of an undifferentiated fate. In general, our experience is that culture conditions that may be rate limiting for maintaining undifferentiated growth of hES cells include MEF quality and density, changes in the osmolarity, pH, and temperature of the medium, as well as the presence of supplements, such as β-mercaptoethanol.

The stable maintenance of diploid chromosome number in the majority of our cell lines indicates that our cell lines maintained a stable karyotype in vitro after extensive passaging and repeated freezing/thawing cycles. The fact that two of the stem cell lines were chromosomally abnormal is not surprising taking into account that *Hardarson et al.* [25] recently found that only 42% of surplus IVF embryos were chromosomally normal at the blastocyst stage.

Consistent with previous reports, xenografting of most of our cell lines (SA002, AS034, AS034.1, SA118, and SA121) generated solid teratomas consisting of endodermal, mesodermal, and ectodermal cell type derivatives [1, 2, 5]. However, some of the cell lines (FC018, AS038) preferentially generated fluid-filled cyst-like structures of human origin. We have yet to determine the cellular identity and origin of these cysts. Potentially, these cells may be of trophoectoderm origin, since it was recently shown that hES cells are capable of differentiating into extraembryonic derivatives, such as trophoblast cells [26]. Alternatively, FC018 and AS038 may represent primitive endoderm lineages. However, the facts that undifferentiated colonies of these cell lines expressed significant levels of Oct-4, and that they lacked expression of primitive endoderm markers, such as Gata4 and AFP [27, 28], suggest that it is unlikely that they are of extraembryonic or primitive endoderm origin (data not shown).

Needless to say, more knowledge is needed about basic hES cell biology, such as the regulatory pathways that govern self-renewal and differentiation, before it will be possible to appreciate the potential applications of hES cells in basic science and cell-replacement therapy.

ACKNOWLEDGMENTS

We acknowledge *Katarina Andersson, Karin Axelsson,* and *Angelica Niklasson* for assistance with derivation,

characterization, and propagation of hES cells; *Anita Sjögren* and *Thorir Hardarson* for culture of human embryos; *Gunilla Caisander* for karyotyping and FISH analysis; *Inger Bryman* and staff at the IVF clinic at the Sahlgrenska University Hospital; *Monalill Lundqvist* at IVF clinic at the Uppsala University Hospital; and *Matts Wikland* at Fertility Center Scandinavia for supplying embryos. We thank *Marie Rehnström* and *Ulrika Karlsson* for assistance in subcloning; *Gabriella Brolén* for support in stainings and cell culture; and *Peter Sartipy* for general assistance. The work was supported by grants from the Cell Therapeutics Scandinavia AB, Swedish Research Council (*H.S.*), Juvenile Diabetes Research Foundation (*H.S.*), and Inga Britt och Arne Lundbergs Forskningsstiftelse (*H.S.*).

References

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-1147.

2 Reubinoff BE, Pera MF, Fong CY et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000;18:399-404.

3 Bongso A, Fong CY, Ng SC et al. Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 1994;9:2110-2117.

4 Piedrahita JA, Anderson GB, BonDurant RH. On the isolation of embryonic stem (ES) cells: comparative behavior of murine, porcine, and ovine embryos. Theriogenology 1990;34:879-901.

5 Amit M, Carpenter MK, Inokuma MS et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000;227:271-278.

6 Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. Hum Reprod 1999;14:3069-3076.

7 Bergh C, Howles CM, Borg K et al. Recombinant human follicle stimulating hormone (r-hFSH; Gonal-F) versus highly purified urinary FSH (Metrodin HP): results of a randomized comparative study in women undergoing assisted reproductive techniques. Hum Reprod 1997;12:2133-2139.

8 Dokras A, Sargent IL, Barlow DH. Human blastocyst grading: an indicator of developmental potential? Hum Reprod 1993;8:2119-2127.

9 Hogan B, Beddinton R, Costatini F et al. Manipulating the Mouse Embryo: A Laboratory Manual. 2nd Ed. Plainview, NY: Cold Spring Harbor Laboratory Press, 1994:260-261.

10 Thomson JA, Marshall VS. Primate embryonic stem cells. Curr Top Dev Biol 1998;38:133-165.

11 McEachern MJ, Krauskopf A, Blackburn EH. Telomeres and their control. Annu Rev Genet 2000;34:331-358.

12 Bosma MJ, Carroll AM. The SCID mouse mutant: definition, characterization, and potential uses. Annu Rev Immunol 1991;9:323-350.

13 Mitalipova M, Calhoun J, Shin S et al. Human embryonic stem cell lines derived from discarded embryos. Stem Cells 2003;21:521-526.

14 Thomson JA, Kalishman J, Golos TG et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 1995;92:7844-7848.

15 Andrews PW, Casper J, Damjanov I et al. Comparative analysis of cell surface antigens expressed by cell lines derived from human germ cell tumours. Int J Cancer 1996;66:806-816.

16 Knowles BB, Aden DP, Solter D. Monoclonal antibody detecting a stage-specific embryonic antigen (SSEA-1) on preimplantation mouse embryos and teratocarcinoma cells. Curr Top Microbiol Immunol 1978;81:51-53.

17 Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell 1990;60:585-595.

18 Hansis C, Grifo JA, Krey LC. Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. Mol Hum Reprod 2000;6:999-1004.

19 Lebkowski JS, Gold J, Xu C et al. Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications. Cancer J 2001;7 (suppl 2):83-93.

20 Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971-974.

21 Itskovitz-Eldor J, Schuldiner M, Karsenti D et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 2000;6:88-95.

22 Vescovi AL, Parati EA, Gritti A et al. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 1999;156:71-83.

23 Solter D, Knowles BB. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci USA 1975;72:5099-5102.

24 Talbot NC, Powell AM, Rexroad CE Jr. In vitro pluripotency of epiblasts derived from bovine blastocysts. Mol Reprod Dev 1995;42:35-52.

25 Hardarson T, Caisander G, Sjogren A et al. A morphological and chromosomal study of blastocysts developing from morphologically suboptimal human pre-embryos compared with control blastocysts. Hum Reprod 2003;18:399-407.

26 Xu RH, Chen X, Li DS et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 2002;20:1261-1264.

27 Dziadek MA, Andrews GK. Tissue specificity of alpha-fetoprotein messenger RNA expression during mouse embryogenesis. EMBO J 1983;2:549-554.

28 Arceci RJ, King AA, Simon MC et al. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol 1993;13:2235-2246.

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From: catharina.ellerstrom@cellartis.com
To: HESCREGISTRY (NIH/NIDCD)
Cc: johan.hyllner@cellartis.com
Subject: SV: New hESC Registry Application Request #2010-ADM-005
Date: Wednesday, June 30, 2010 12:59:44 PM
Attachments: Document 4. Assurance of Conditions SA001, SA002, SA002.5.pdf
ENCLOSURE 1 Procedure for establishment.pdf
Document 2. Informed Consent.pdf
Document 3 Certification of signed informed consent.pdf

Dear Dr Gadbois,

Fist of all I would like to apologize for our delay in response in this matter. This is not in any way reflecting our intension/aim to have our lines added to the NIH Human Embryonic Stem Cell Registry which is highly important to us. Below you will find our answers on your questions regarding our hES lines SA001, SA002 and the SA002.5. As discussed with you colleagues that attended ISSCR 2010 we send you these answers in an e-mail, but if you need the content as a signed letter we will be happy to provide that to you. If you have any further questions or need of clarifications please do not hesitate to ask me. I will answer promptly.

1. Can you confirm that the cell lines from which SA001 and SA002 were derived were considered to be of "low quality" (i.e. not appropriate for uterine transfer)?
Yes, we can:
According to Swedish law surplus embryos can not be stored frozen longer than up to five years. Consequently, embryos that have been stored for 5 years can not be used for uterine transfer and are then to be discarded. These embryos can be used for scientific purposes, e.g. to derive hESCs, if the individuals who sought reproductive treatment gave a voluntary written consent. This means that in Sweden we are allowed to not only derive hESCs from embryo of low quality" but also from blastocysts originating from high quality embryos that had been stored for 5 years. Consequently, blastocysts were obtained from the IVF clinic that either came from poor developed embryos (i.e. not appropriate for uterine transfer) or from surplus embryo that had been frozen but kept in the liquid nitrogen tank for 5 years. These embryos were then cultured to blastocysts from which hESCs could be derived. *

SA002 was derived from an embryo that had been stored in liquid nitrogen for the maximum time and was therefore excluded for any uterine transfer. SA001 was derived early on and the information of this blastocyst is in a laboratory book from early 2001 by a person who long ago left the company. If this information is vital for NIH Human Embryonic Stem Cell registry, then I will definitely revisit our archive again. Please let me know! *Nota Bene*! Both SA001 and SA002 was derived from blastocysts according to NIH Guidelines Section II(A). *
Enclosure 1 describes the whole procedure for hESCs establishment and includes a description of the donating procedure as well.

2. Can you please confirm whether both members of the donor couple signed the informed consent forms? Please note that the NIH Guidelines require written information consent from the individual(s) who sought reproductive treatment, so in the case of a couple who used a third party gamete donor, both members of the couple must still consent for the donation of the embryo for research
According to Swedish law both members of the donor couple must sign the written consent. We hereby confirm that written consent was obtained from the both members of the donor couple from SA001 and SA002. This is reflected in all provided expamples of donor consents (see Enclosure 2) with lines for both donors to sign. In the later donor consents (end of Enclosure 2) this is clarified further since also both donors have to provide their social security numbers. Finally, in Enclosure 3, Professor Christina Bergh confirms that written consent has been obtained from both donating couples. *

Please provide further detail on how Element 6 of the NIH Guidelines was met. ("Decisions related to the creation of human embryos for reproductive purposes should have been made free from the influence of researchers proposing to derive or utilize hESCs in research. The attending physician responsible for reproductive clinical care and the researcher deriving and/or proposing to utilize hESCs should not have been the same person unless separation was not practicable.") In particular, please provide:

The name of the individual(s) who served as the treating physician(s) at the fertility treatment facility for the patients who donated the embryos from which SA001 and SA002 were

derived.
Associate Professor Lars Nilsson served as the attending physician at the time of the donations.
Was the physician(s) also involved in the research to derive or utilize hESCs from those embryos? No he was <u>not</u> involved in the research to derive or utilize hESCs from those embryos.
The name of the principal investigator(s) for the research project. Did the principal investigator(s) interact with the embryo donors? Associate Professor Charles Hanson was the PI for the research project. He had <u>no</u> whatsoever contact with the donating couples.
See also Enclosure 1 which describes the donor consent process.
See also Enclosure 4 which shows assurance of conditions of consent for human embryonic stem cell lines SA001, SA002 and SA002.5.

Please provide further detail on how Element 8 of the NIH Guidelines was met. ("Donor(s) should have been informed that they retained the right to withdraw consent for the donation of the embryo until the embryos were actually used to derive embryonic stem cells or until information which could link the identity of the donor(s) with the embryo was no longer retained, if applicable.") We note that donors were informed by the Patient Information sheet that they could terminate their participation <u>at any time</u>. Were donors provided any other information (written or oral) indicating that there was a point at which donation of the embryo could not be revoked? For lines that <u>can</u> be identified the donating couple can terminate their participation at any time. For the non-identified lines (which include SA001, SA002 and SA002.5) the donating couples could terminate their participation until the information which could link the identity of the donors with the embryo (hESC line) was no longer retained. The donors obtained this information.

Please provide further detail on how Element 10 of the NIH Guidelines was met, either in written information or through oral discussions. ("During the consent process, donor(s) were informed of the following: What would happen to the embryos in the derivation of hESCs for research.")
iis information has been provided to the donating couples in written (please see Enclosure 2).

Please provide further detail on how Element 11 of the NIH Guidelines was met, either in written information or through oral discussions. ("During the consent process, donor(s) were informed of the following: That hESCs derived from the embryos might be kept for many years.") We note that the donors were reconsented several times for use of the hESC lines for certain periods of time. Please clarify what the donors were told on this topic. It is correct that the donors were reconsented several times. This reflects in many ways the development of the hESC field. Both SA001 and SA002 were derived early 2001 when the human stem cell field was new to the world and to Sweden. When the first donor consents were prepared there was yet no practical experience of hESC derivation and culture in Sweden and the possible donors were only asked if their surplus embryos could be used to develop the hESC culture technique. In the 2nd written consent we asked to extend the culture time to 2 years. In the 3rd consent we asked for their permission to extend the culture beyond 2 years and in the 4th we asked for their permission to send the hESCs abroad to other research laboratories.

Please provide further detail on how Element 15 of the NIH Guidelines was met, either in written information or through oral discussions. ("During the consent process, donor(s) were informed of the following: Whether information that could identify the donor(s) would be available to <u>researchers</u>.")
:llartis do not have, and have never had any whatsoever possibility to identify the donating couples. Information and informed consent was handled by an attending physician (Associate Professor Lars Nilsson) who had no involvement in the stem cell project. Initially he orally informed the donors that <u>only</u> key personal at the IVF clinic were able to connect donors to embryo, but in the later consents this information was also included written (see Enclosure 2).
)u will find the whole donating procedure described in Enclosure 1 and also a more detailed answer in Enclosure 4.

Kind regards,

Catharina

cellartis

Procedure for the establishment of human embryonic stem cell lines

Location: Sahlgrenska University Hospital
Dept of Obstetrics and Gynaecology
Göteborg, SWEDEN

An IVF-treatment is usually preceded by an infertility investigation, which includes e.g. ultrasound of uterus and ovaries, investigation of tubal patency, hormone and sperm analysis. Normally, several eggs are fertilized during IVF and if the surplus embryos which are not transferred are considered to be of adequate quality (see below) they can be stored frozen. If the woman does not become pregnant with the fresh eggs, the frozen eggs can be thawed and introduced at a later time point. The embryos that do not reach the stringent inclusion criteria for transfer or freezing are discarded (Grade 3 or 4 embryos). According to the Swedish law, fertilized eggs can be stored frozen for a maximum of 5 years. After this period the embryos can not be used for implantation and are therefore discarded. Patients can, if they wish, finish the storage whenever they like during this 5-year period and decide that the embryos should be discarded.

However, it is possible to continue culturing these embryos that otherwise should have been discarded. Some of these embryos will thereby develop to blastocysts from which the inner cells mass can be isolated and be the source of embryonic stem cells. A prerequisite for using these embryos for research is that the patients give their informed consent.

For donation of fresh embryos, the patients are informed about the project during the first visit at the clinic before the start of the IVF-treatment. This gives the patients 1-2 months to consider their decision regarding donation of embryos.

For donation of frozen embryos, the patients are informed by a letter about the project well before the 5-year time limit for frozen embryos. If they wish to donate their frozen embryos, they sign and return an informed consent.

Procedure for fresh embryo:
On day 0 the oocyte is aspirated and fertilized. The fertilized and cleaved oocyte, i.e. the embryo, is evaluated based on morphology and cell division on day 2. The following scale is used for embryo evaluation:
Grade 1 embryo: 4-6 cells, even sized blastomeres, no fragments
Grade 2 embryo: 4-6 cells, <20% fragments and/or uneven sized blastomers
Grade 3 embryo: >20% fragments and/or degenerated cells
Grade 4 embryo: >50% fragments and or degenerated cells

After evaluation on day 2, embryos of grade 1 and 2 are either transferred or frozen for transfer at a later time point. Embryos of grade 3 and 4 are discarded.

Procedure for freezing of embryos:
At day 2 after fertilization the embryos are frozen at the 4-cell stage.
Frozen embryos are stored in liquid nitrogen.

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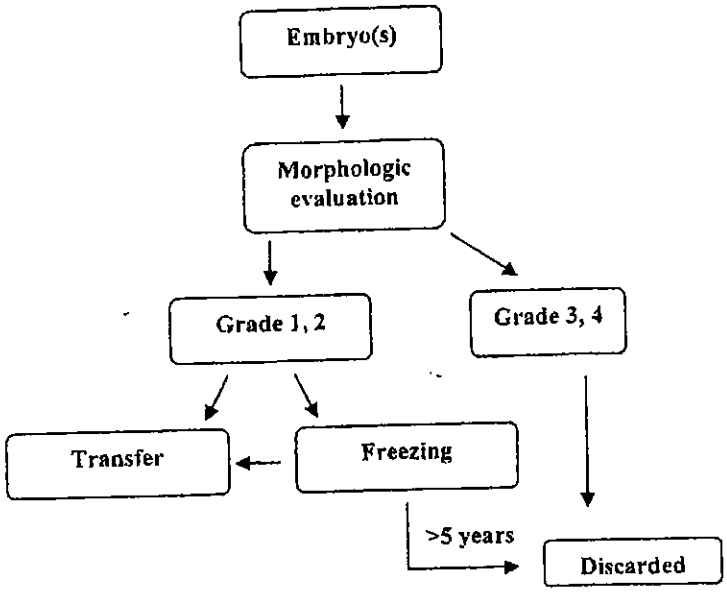

Figure 1. Flow chart illustrating the procedure for selection of embryos used for transfer.

It is possible to isolate embryonic stem cells from blastocysts established from discarded embryos (either fresh or frozen) initially produced for IVF treatment if informed consent has been obtained from the patients.

Procedure for fresh embryo:
Cleavage state embryos of grade 3 are donated day 2 after fertilization. The embryos are further cultured for 2-4 days (i.e. to day 5-7). These later stage embryos, i.e. blastocysts are evaluated according to the following scale:
Grade A Blastocyst: Expanded with distinct inner cell mass (ICM) on day 5 or 6
Grade B Blastocyst: Poorly expanded and/or less defined ICM
Grade C Blastocyst: Poorly developed with no visible ICM or with degenerative foci in the ICM

Procedure for frozen embryo:
At day 2 after fertilization the embryos are frozen at the 4-cell stage and stored in liquid nitrogen. The embryos are thawed, and the procedure described above is then followed.

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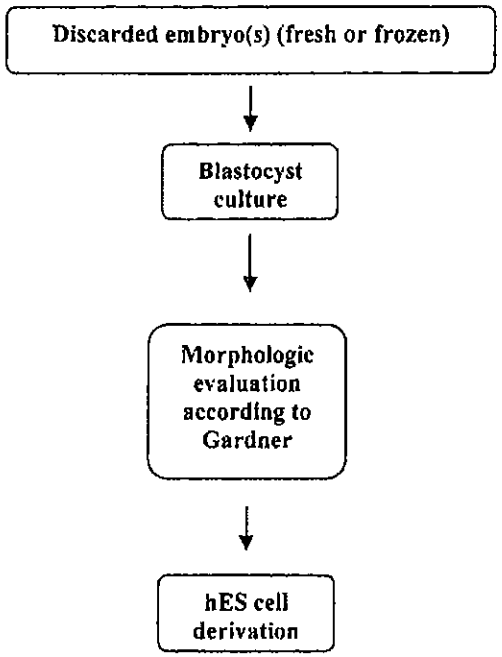

Figure 2. *Flow chart illustrating the procedure for establishment of blastocysts used for derivation of hES cells. See text for details.*

Storage and information flow
The undertaken research will solely use existing data or specimens. In addition, the data or specimens are not publicly available and the information recorded by the investigators cannot be directly linked to the subject. Thus, it does not involve individually identifiable private information such that the identity of the donors is or may readily be ascertained by the investigator or associated with the information.

Patients enrolled in the in vitro fertilization (IVF) program at the Sahlgrenska University Hospital, Gothenburg, Sweden, are informed of the stem cell project by a physician who is not directly connected to the stem cell project. If the couple signs an informed consent all their surplus embryos (i.e. embryos not transferred to the woman or frozen) are cultured further, to the blastocyst stage. The actual procedure renders no financial gain for the donating couple no matter the extent of physiological and/or psychological suffering that be the result of the IVF treatment. The embryo selection for the hES cell project is performed by independent laboratory technicians.

If the donated embryos reach the blastocysts stage (at day 5-7 after fertilization), they are given a number, not linked to the patient and transported to a different facility where the ES cell lines are established, figure 3. The records are kept in two separate databases. One database has all the information regarding culture conditions and results of the embryo culture and the blastocyst number (which if established, also becomes the human ES line number) but no information regarding the patient. For the unlinked anonymized lined no connection exists between those two databases, figure 4.

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Furthermore, according to Swedish law, the IVF number cannot be used to identify the patient's identity without access to database I which has access codes only supplied to authorized hospital personnel working at the IVF clinic and again, are unrelated to the stem cell project. The above described procedures are summarized in Figure 3-4.

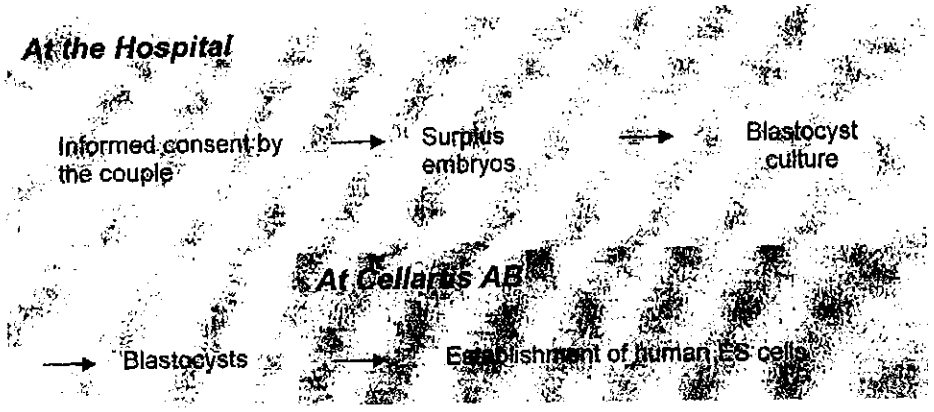

Figure 3. A flow chart showing the handling process with donated embryos.

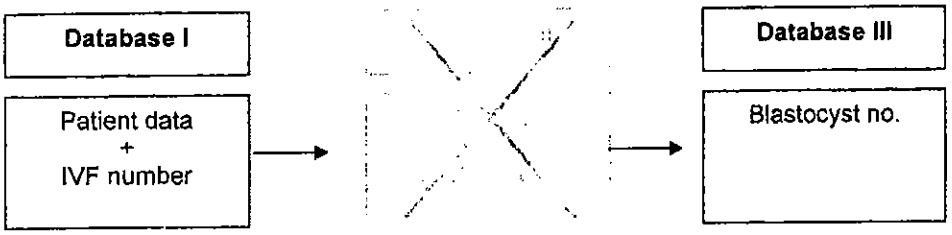

Figure 4. A flow diagram showing how information for the unlinked anonymized hESC lines are stored separately in two different databases. There is no connection between the two databases and persons with access to database III do not have access to database I.

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This is to certify that the above described procedure is correct and in accordance with national legislation and ethical guidelines:

Name and position:
Johan Hyllner, COO, Cellartis AB

2006-11-07.
Date:

Name and position:
Christina Bergh, MD, professor,
Dept of Obstetrics and Gynecology,
Sahlgrenska University Hospital

2006-11-02
Date:

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From: catharina.ellerstrom@cellartis.com
To: HESCREGISTRY (NIH/NIDCD)
Cc: johan.hyllner@cellartis.com
Subject: SV: New hESC Registry Application Request #2010-ADM-005
Date: Tuesday, July 13, 2010 9:41:03 AM
Attachments: Signature of signing official SectionIIB .pdf

Dear Dr. Hannemann,

I'll start with your last question regarding a clarification of the text in the translated version of the consent form, Appendix iii: The first sentence "**"We permit that our cells are cultured up to 2 years."** was not corrected translated. It should read "**We permit that our cells are cultured further for two additional years**.".The 2nd phrase is correct translated. I apologize for this mistake.

Attached please find the signed IIB assurance letter. If you need the original, please let me know.

To be able to answer your three questions in the email from 9th of July I need to obtain the required information from the IVF-clinic. Unfortunately they are presently on summer vacation, but as soon as they return I'll ask them to provide us with the information.

Kind regards,

Catharina Ellerström

Från: HESCREGISTRY (NIH/NIDCD) [mailto:hescregistry@mail.nih.gov]
Skickat: den 12 juli 2010 17:57
Till: 'catharina.ellerstrom@cellartis.com'
Kopia: johan.hyllner@cellartis.com; HESCREGISTRY (NIH/NIDCD)
Ämne: RE: New hESC Registry Application Request #2010-ADM-005

Dear Dr. Ellerstrom,

In addition to the questions below, we hope you can clarify the following. In the translated version of the consent form, Appendix iii, please clarify the meaning of the two options presented:

"We permit that our cells are cultured up to 2 years."

"We do not permit that our cells are cultured more than 2 years."

From the English interpretation, it appears that either choice results in cells being cultured for up to 2 years. Please clarify what is being asked of the donors and the differences in the two options.

Thank you for attention to our inquiries.

Sincerely,

-Diane Hannemann

Diane E. Hannemann, Ph.D.
Office of Science Policy Analysis
Office of the Director
Bldg 1, Room 218D
National Institutes of Health
voice: 301.594.0064
fax: 301.402.0280

* original text on p. 20

(68)

From: Catharina Ellerstrom
To: HESCREGISTRY (NIH/OD)
Subject: RE: New hESC Registry Application Request #2010-ADM-005
Date: Tuesday, July 27, 2010 11:57:27 AM

Dear Dr. Hanneman,

1. For you first question I have asked our legal people to clarify the text, but I have not yet received any answer and I apologize for this delay.
2. SA001 was made from a cryopreserved embryo that had reached the 5 years storage limit. The embryo was created at the IVF clinic with the intention to be re-implanted into the woman, in uterus.
3. I do not know the rationale behind the 5 years storage limit, but I will forward this question to the IVF clinic. They should return back from their vacations shortly.

*

Kind regards,

Catharina

-----Original Message-----
From: "HESCREGISTRY (NIH/NIDCD)" <hescregistry@mail.nih.gov>
To: "'catharina.ellerstrom@cellartis.com'" <catharina.ellerstrom@cellartis.com>
Cc: "johan.hyllner@cellartis.com" <johan.hyllner@cellartis.com>, "HESCREGISTRY (NIH/NIDCD)" <hescregistry@mail.nih.gov>
Date: Tue, 20 Jul 2010 11:24:42 -0400
Subject: RE: New hESC Registry Application Request #2010-ADM-005

Dear Dr. Ellerstrom,

The Working Group for Human Embryonic Stem Cell Eligibility Review has begun its review of this submission and has asked the following questions:

1) Please clarify what is meant by the "provider restriction" stating, "research use only." Does this mean no therapeutic or clinical use, or no commercial use? (This has no bearing on the decision of whether the lines are eligible for use under the NIH Guidelines, but rather is information that we want to make sure is understood correctly by NIH grantees in the event that the lines are approved.)

2) Please confirm that SA001 was derived from a clinical grade cryopreserved embryo.

3) We understand from your submission that according to Swedish law, surplus embryos cannot be stored frozen longer than five years. Do you know the rationale for this requirement?

Thank you for your continued efforts in this review process.

Sincerely,
-Diane Hannemann

Diane E. Hannemann, Ph.D.
Office of Science Policy Analysis
Office of the Director
National Institutes of Health
voice: 301.594.0064
fax: 301.402.0280

(69)

From: Catharina Ellerstrom
To: HESCREGISTRY (NIH/OD)
Subject: RE: New hESC Registry Application Request #2010-ADM-005
Date: Thursday, August 19, 2010 5:04:34 PM
Attachments: ENCLOSURE 1 Donor Consent A (English)1 .pdf
ENCLOSURE 2 .pdf
ENCLOSURE 3 . Assurance ofConditions SA001, SA002, SA002.5.pdf

Dear Dr. Hannemann,

Below find our answers. If you have any further questions we will be ready to answer them too (promptly this time since everybody now are back at work).
Kind regards,

Catharina Ellerström

-----Original Message-----
From: "HESCREGISTRY (NIH/OD)" <hescregistry@mail.nih.gov>
To: "'catharina.ellerstrom@cellartis.com'" <catharina.ellerstrom@cellartis.com>
Cc: "HESCREGISTRY (NIH/OD)" <hescregistry@mail.nih.gov>
Date: Tue, 17 Aug 2010 10:49:59 -0400
Subject: RE: New hESC Registry Application Request #2010-ADM-005

Dear Dr. Ellerstrom,

The NIH Advisory Committee to the Director Working Group for Human Embryonic Stem Cell Eligibility Review is meeting next week. We would like to include the Cellartis submission (now numbered 2010-ACD-010) on the agenda for discussion, if possible.

To do so, please let us know if you can obtain the information you requested from the IVF clinic and send to NIH by this Thursday, August 19th (USA calendar). Below is a recap of the open questions. Information regarding the first question would be most helpful before the working group meeting next week.

1) How were patients informed of the options available for use of the embryos no longer needed for reproductive purposes? In particular, please provide a copy of the letter (mentioned in the document titled "Procedure for the establishment of human embryonic stem cell lines") which was given to patients with frozen embryos prior to the 5-year time limit for retention of frozen embryos. Both SA001 and SA002 were derived from frozen embryos that had reached their frozen storage limit of 5 years. The patients recived a letter well in advance of the deadline in where they where informed of the different choices they had. You will find the translated content in Enclosure 1 and the original in Swedish in Enclosure 2. *

2) Please send copies of the IVF treatment consents, with any patient information removed. See Enclosure 3 inwhere the head of the IVF clinic state and signed a document describing the IVF treatment and the donating procedure. I do not belive that there was any specific IVF treatment concent since this should not be necessary in Sweden according to the Swedish law. Please let me know if this information is sufficient for the NIH or if you need further clarifications. see * p.70

3) Please clarify what is meant by the "provider restriction" stating, "research use only." Does this mean no therapeutic or clinical use, or no commercial use? (This has no bearing on the decision of whether the lines are eligible for use under the NIH Guidelines, but rather is information that we want to make sure is understood correctly by NIH grantees in the event that the lines are approved.) This text is found in our MTA for hES cells. The hES cell lines SA001 and SA002 are **not** approved for theraputical or clinical use and our MTA do not permit any commercial use. *

SAHLGRENSKA UNIVERSITY HOSPITAL

Division of Women's Health Care, Urology and Oncology

2001-04-09

You have undergone IVF treatment .....and at that time .....numbers of embryos were frozen down. The time for your storage of frozen embryos will expire within 3-6 months, more specifically the ..... and according to our notes you have not applied for exemption (for longer storage). If no application for exemption is submitted prior the storage deadline then according to the Swedish law we have to destroy your embryos. Since the freezing/storage time is as long as five years, we believe that Socialstyrelsen (The National Board of Health and Welfare) will not grant an exemption as long as not very specific reasons are presented.

An application to Socialstyrelsen shall according to their instructions, be submitted latest two months prior to the expiration of the freeze storage time. The application should be submitted to us two weeks prior so that we can confirm the presence of the frozen embryos.
To facilitate any application for exemption, please state clearly if you have any specific reasons or cause to apply for an exemption.

If we do not receive any exemption application from you prior the stated frozen storage deadline then we will follow the Swedish law and destroy your embryos.

In the event of that you do **not** apply for an exemption we would be very grateful if you could inform us in time and that you inform us if you will allow method development/ research on the embryos prior their destruction or if you do not allow any work to be conducted using your embryos. Please indicate your answer in one of the boxes below. If we get your permission to use your embryos for method development /research then we kindly ask you to read through and sign the attached patient information. The signed patient information form should then be returned back to us together with this signed document (a stamped and addressed envelope is attached).

Göteborg 2001-02-19

Lars Nilsson,
Associate Professor
Division of Women's Health Care/IVF
SU/Sahlgrenska

☐ Are applying for an exemption (please attach copy).

☐ Are **not** applying for exemption. You may destroy our embryos.

☐ Are **not** seeking exemption. We allow research using the embryos prior to destruction (please attach the signed patient information).

Date.....
Signatures.....

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From: catharina.ellerstrom@cellartis.com
To: HESCREGISTRY (NIH/OD)
Subject: SV: New hESC Registry Application Request #2010-ADM-005
Date: Tuesday, August 24, 2010 10:00:32 AM

Dear Dr. Hannemann,

According to the information I have obtained from the IVF people we work with, the following arguments were behind the Swedish legislation that limits the storage of human embryos to five years: First and most importantly they wanted to avoid a too big age gap between siblings. Then, there was also a practical reason to limit the storage time. An unlimited or a longer maximum storage time would demand significantly more storage space and handling. *

Kind regards,

Catharina Ellerström

Från: HESCREGISTRY (NIH/OD) [mailto:hescregistry@mail.nih.gov]
Skickat: den 20 augusti 2010 18:16
Till: 'catharina.ellerstrom@cellartis.com'
Kopia: HESCREGISTRY (NIH/OD)
Ämne: RE: New hESC Registry Application Request #2010-ADM-005

Dear Dr. Ellerstrom,

Thank you for this additional information. I hope you may also be able to answer this earlier question from the Working Group:

> We understand from your submission that according to Swedish law, surplus embryos cannot be stored frozen longer than five years. Do you know the rationale for this requirement?

Thank you for your on-going efforts as we continue this review process.

Sincerely,
-Diane Hannemann

Diane E. Hannemann, Ph.D.
Office of Science Policy Analysis
Office of the Director
National Institutes of Health
voice: 301.594.0064
fax: 301.402.0280

From: Catharina Ellerstrom [mailto:catharina.ellerstrom@cellartis.com]
Sent: Thursday, August 19, 2010 5:04 PM
To: HESCREGISTRY (NIH/OD)
Subject: RE: New hESC Registry Application Request #2010-ADM-005

Dear Dr. Hannemann,

Below find our answers. If you have any further questions we will be ready to answer them too (promptly this time since everybody now are back at work).
Kind regards,

Catharina Ellerström