BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE DEPÓSITO DE UNA LÍNEA
Application Form to Deposit a Human Cell Line

Documentos que se acompañan:
Attached documents:

☒ Copia de la autorización de derivación de la línea celular, junto con informe del Comité Ético del centro de procedencia.
A copy of the authorization for the derivation of the cell line, with the corresponding ethics committee approval

☒ Copia de cualquier publicación científica relacionada con la derivación y/o caracterización de la línea.
A copy of any relevant published scientific papers related to the derivation and/or characterization of the cell line

☒ C. V. del investigador principal (una página; formato libre).
A one page CV for the Principal Investigator

☒ Otros (especificar).
Others (specify)

Anexo 1:Tipado HLA y microsatélites.
HLA and STR analyses

Anexo 2: Secuencia de imágenes de la evolución del embrión descongelado hasta colonia primaria.
Representative images of the thawed embryo throughout the embryonic outgrowth development

Anexo 3: Informe del análisis microbiológico.
Microbiologiy Testing

Anexo 4: Informe del análisis de micoplasma.
Mycoplasm Testing

Anexo 5: Caracterización fenotípica
Immunephenotypic characterization

Anexo 6: Factores de Trascripción y Cariotipo (Bandeo G).
Expression of transcription factors and Karyotype G-Banding analyses

Anexo 7: Diferenciación *in vitro*.
In vitro differentiation

Anexo 8: Diferenciación *in vivo*.
In vivo differentiation

Anexo 9: Copia de la publicación (sometida)
Copy of the submitted manuscript.

SECCIÓN 1
Section 1

Información General
General Information

Nombre de la línea: GRA-1
Name of the line:

Investigador principal: PABLO MENENDEZ BUJAN/JOSE LUIS CORTES ROMERO
Principal Investigator:

Origen de la línea celular:
Origin of the cell line

Embrionario ☒ Fetal ☐ Adulto ☐
Embryonic *Fetal* *Adult*

¿La línea celular ha sido derivada de un embrión con anomalía genética?
Has the cell line been derived from an embryo with genetic anomaly?

NO ☒ SÍ ☐ (especificar)
No *Yes* *(specify)*

Identificación genética de la línea celular. Método y resultado
Genetic identity of the cell line. Method and result

Análisis de microsatélites (ver anexo 1)
Microsatellite characterizaction (see Annex 1)

SECCIÓN 2
Section 2

Datos del Depositante
Applicant Details

Investigador Principal: *Principal Investigator:* Pablo Menendez Buján J.L Cortés Romero	Dirección Postal: *Postal address:* Avda. Conocimiento s/n. Centro de Investigación Biomédica. 18100. Armilla (Granada)
Centro de Trabajo: *Institution:* BANCO ANDALUZ DE CÉLULAS MADRE Andalusian Stem Cell Bank	Teléfono *(phone)*: +34 958 894 672 Fax: +34 958 894 652 E-mail: pablo.menendez@juntadeandalucia.es

Tipo de muestra biológica (especificar estadio embrionario, semanas de gestación,...) *Kind of biological sample (specify embryonic stage, weeks of pregnancy,...)* Embrión humano en estadio de blastocisto Blastocyst-stage human embryo
Muestra biológica *Biological sample* Fresco ☐ *Fresh* Crioconservado ☒ *Cryopreserved*

Fecha de la obtención del muestra biológica *Date of obtaining the biological sample* July 1999	Fecha del uso o descongelación *(si congelado)* *Date used or thawed (if frozen)* 9.6.2008
Fecha de la donación del muestra biológica *Date of donation of the biological sample* 12.12.2007	

Descripción general del procesamiento previo del muestra biológica utilizado (cultivo embrionario, procesamiento muestra fetal o de tejido adulto)
General description of the processing of the biological sample used (embryonic culture, processing of fetal sample or of adult tissue)

El embrión crioconservado donado fue descongelado en junio de 2008 mediante un protocolo de descongelación lenta utilizando glicerol y sacarosa. El embrión estaba congelado en día +6 desde el mes de julio de 1999. Tras 2 días de cultivo en medio secuencial especial para cultivo embrionario, suplementado con el inhibidor de ROCK Y-27632, se eliminó la zona peluzida utilizando Ácido Tyrode. El embrión se colocó directamente sobre una monocapa de células mesenquimales humanas (hMSCs) irradiadas y medio de cultivo de células madre embrionarias humanas (hESCs). Tras 4 días de cultivo, se apreció en la colonia primaria una zona que se correspondía con el aspecto característico de la masa celular interna (ICM). Se procedió entonces a la destrucción de las células del trofoblasto mediante el empleo de un disector láser para poder aislar la ICM, que fue subcultivada mecánicamente a una nueva placa con hMSCs ($0.5x10^5$ cells/cm^2). (Anexo 2)

The donated frozen embryo was thawed last June 2008 using a slow-freezing method with glycerol and sacarose. The embryo was initially frozen at day 6 of development. After 2 days in culture with sequential embryo culture media, G-1 v.5 and G-2 v.5 (Vitrolife) supplemented with the ROCK inhibitor Y-27632, the zona pellucida was removed with Tyrode´s Acid. The embryo was then placed on a feeder layer of irradiated human mesenchymal stem cells and hESC media. Four days later, the inner cell mass could be detected. The trophoectoderm was disrupted by means of laser-assisted technology releasing the ICM. ICM outgrowths were allowed to expand further. The first ESC colonies started to show up and were subsequently subcultured on fresh irradiated human mesenchymal stem cells ($0.5x10^5$ cells/cm^2) and hESC media (Annex 2).

En caso de muestra embrionaria, indicar si se utilizaron blastómeros o células de la masa celular interna y el método de aislamiento utilizado
If of embryonic origin, indicate whether blastomeres or internal cell mass were used, as well as the isolation method

El blastocisto completo desprovisto de zona pelúcida fue colocado sobre hMSCs, aislando posteriormente la ICM mediante el empleo del láser (Anexo 2).

The whole blastocyst was seeded on hMSCs upon removal of the zona pellucida. The ICM was isolated using laser-assisted whole embryo culture (annex 2).

Origen del soporte celular o acelular utilizado para la derivación, así como de los componentes de los medios de cultivo (si se describen en publicación, indicar además referencia)
Origin of the cellular or cellular free support used in derivation in addition to the components of the culture mediums (if they are described in a publication, please indicate the reference).

Support: human mesenchymal cells cultured in IMDM and advanced-DMEM, respectively, plus 10% FCS and 2mM L-glutamine.
Culture medium: KO-DMEM supplemented with 20% KO Serum Replacement, 1% non-essential amino acids, 1mM L-glutamine, 0.1 mM β-mercaptoethanol and 8ng/mL of bFGF (all from Invitrogen, CA)

Mantenimiento de la línea: *Line maintenance*
Ratio de pase: *Passage ratio:* 1:2-1:3 cada 5-7 días; 1:2-1:3 every 5-7 days
Método de pase: *Passage method:* **método mecánico y enzimático;** either mechanical or enzymatic

Xenobióticos *Xenobiotics*	**si X** *Yes*	**no** *No*

Descripción de las características morfológicas de la línea en cultivo
(forma y tamaño colonias; forma y tamaño células; ratio núcleo/citoplasma; otros)

Description of the morphological characteristics of the line in culture (form and size of the colonies; form and size of the cells; nucleus/cytoplasm ratio; others)

El aspecto de las colonias es la típica de las hESCs: redondeado, aplanado y uniforme. Las colonias son grandes y sin bordes lisos. Alta relación nucle/citoplasma. (Anexo 2).

Gra-1 showed the typical hESC morphology: round and flat colonies of uniform size. The colonies are medium-large size with well-defined edges. There is a high nucleus/cytoplasm ratio (Annex 2).

Controles microbiológicos realizados (indicar detalladamente)

Microbiological controls carried out (indicate in detail)

Los estudios externos de micología y micoplasma son negativos tras 26 pases (Anexo 3 y 4)

Mycology and mycoplasm testing proved to be negative (Annex 3 & 4).

Marcadores (Anexos 5 & 6)
Markers

	Método (ARN/proteínas) *Method (RNA/proteins)*	nº pase *Passage n.*	resultado *results*	comentarios *comments*
Oct 4	**RT-PCR**	**7**	**+**	**(Anexo 6)**
Nanog	**RT-PCR**	**7**	**+**	**(Anexo 6)**
Rex 1	**RT-PCR**	**7**	**+**	**(Anexo 6)**
Sox 2	**RT-PCR**	**7**	**+**	**(Anexo 6)**
SSEA3	**Inmunofluoresc/citom flujo**	**7**	**+**	**(Anexo 5)**
SSEA4	**Inmunofluoresc/citom flujo**	**7**	**+**	**(Anexo 5)**
TRA-1-60	**Inmunofluoresc/citom flujo**	**7**	**+**	**(Anexo 5)**
TRA-1-81	**Inmunofluoresc/citom flujo**	**7**	**+**	**(Anexo 5)**
Fosfatasa Alk.	**Actividad**	**7**	**+**	**(Anexo 5)**
Cariotipo / *Karyotype*		**7**	**46, XY**	**(Anexo 6)**
Otros / *Others*				

Capacidad de diferenciación (Anexos 7 & 8)
Differentiation capacity

In vitro (Anexo 7). Formación de cuerpos embrionarios

Presencia de linajes de las tres capas germinales.
Presence of tissues representing the three germ layers.

In vivo (Anexo 8) **Método: Formación de teratomas en ratones NOD/SCID** **Resultado:+**
Method: Teratoma formation in NOD/SCID mice. Result:+

Ectodermo/ *Ectoderm*			**Endodermo**/*Endoderm*			**Mesodermo**/ *Mesoderm*		
marcador *marker*	**pase** *passage*	**resultado** *result*	**marcador** *marker*	**pase** *passage*	**resultado** *result*	**marcador** *marker*	**pase** *passage*	**resultado** *result*
β-tubulina	**10**	**+**	**α-fetoproteína**	**10**	**+**	**sm-actina**	**10**	**+**
			Pan CK	**10**	**+**			
β-tubulin	**10**	**+**	**α-fetoprotein**	**10**	**+**	**sm-actin**	**10**	**+**
			Pan CK	**10**	**+**			

Descripción de las características de diferenciación *in vitro*
Description of the differentiation characteristics in vitro

Los cuerpos embrionarios se crecieron durante 22 días en presencia de 20% FCS. A continuación se embebieron en parafina y se hicieron immunostainings para α-fetoproteina (endodermo), Actina (mesodermo) y βIII-Tubulina (ectodermo). (Anexo 7).

Near confluent hESCs were treated with collagenase IV for 5 min at 37ºC, transferred ($2x10^2$ cells/cm^2) to non-adherent plates and allowed to differentiate spontaneously by embryoid body (EB) formation in DMEM supplemented with 20% FBS, 1% L-glutamine, 0.1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol with media changes every 4 days. After 21 days of EB differentiation, EBs were spun down, fixed with 4% paraformaldehyde for 10 minutes and embedded in paraffin (Catalina et al., 2008a). For each staining, three sections per specimen were used. Then, the cells were incubated (1 hour at RT) with the primary antibodies anti-α-fetoprotein (Santa Cruz Biotechnology; 1:500 dilution in PBS), anti-β-III Tubulin (Chemicon, 1:100 dilution in PBS) and anti-smooth-muscle actin (Chemicon, 1:100 dilution in PBS). Slides were then incubated with a byotinylated secondary antibody (30 minutes at RT) and a streptavidin peroxidase complex (30 minutes at RT) (both from Vector Laboratories Inc). The immunostaining was visualized using diaminobenzidine and counterstained with hematoxylin. All the washing steps were done in PBS. (Annex 7).

Datos de la determinación de pluripotencialidad *in vivo* o formación de teratomas
Data of the pluripotentiality determination in vivo or teratoma formation

Unas 20-40 colonias fueron transplantadas en el testículo de ratones NOD/SCID. Tras 8-10 semanas los ratones desarrollaron tumores palpables Tras el sacrificio del ratón NOD/SCID, de extrajeron los testículos, se fijaron en formol y se obtuvieron muestras histológicas que se tiñeron con hematoxilina/eosina e IH para la identificación de tejidos pertenencientes a las tres capas germinales. (Anexo 8)

During routine passage, 20-40 clumps consisting of about 100 undifferentiated cells each were harvested and injected into the testis of 6 to 8-week-old NOD/SCIDIL2Rγ$^{-/-}$ mice. Eight to ten weeks later, the resulting teratomas were fixed in 10% neutral buffered formalin, embedded in paraffin, and examined histologically after hematoxylin and eosin staining as previously described (Cortes et al., 2008; Catalina et al., 2008a). (Annex 8)

Datos de la tipificación HLA
HLA typification data
Anexo 1 / Annex 1

Consistencia celular tras 6 pases de congelación y descongelación. Resultados.
Cell consistency alter 6 passages of freezing and thawing. Results.
Las células han sido congeladas y descongeladas en varias ocasiones a lo largo de 28 pases. La viabilidad y consistencia celular es óptima. La congelación se ha realizado mediante el uso de un congelador programable.

Human ESCs survived well to several freeze-thaw procedures throughout 28 passages. Cell viability was high and stability was maintained. A programmed freezer was used to warrant high viability rates.

Pase en el momento del registro
Passage at the time of the recording

Actualmente, la línea se encuentra en pase 28. Existen viales congelados a diferentes momentos.
Gra-1 hESC line has been grown for up to 28 passages. There are ampoules/stocks frozen at different time points.

¿Ha sido la línea modificada genéticamente?
Has the line been genetically modified?

Sí *Yes* ☐ No *No* ☒

Comentarios/ *Comments*:

¿Se llevó a cabo un análisis clonal?
Has a clonal analysis been carried out?

Sí/ *Yes* ☐ No ☒ Resultado / *Result*

Otras observaciones o información relevantes (a juicio del Investigador Principal):
Other observations or relevant information (to the discretion of the Principal Investigator):

La línea embrionaria humana GRA-1 ha sido derivada mediante el método de cultivo directo utilizando para el cultivo embrionario medio de cultivo suplementado con un inhibidor de ROCK, sobre una superficie celular formada por células mesenquimales humanas (hMSC). Esta línea crece actualmente tanto en hMSCs como en Matrigel.

Gra-1 has been derived on MSCs used as feeders. To improve embryo survival the ROCK inhibitor Y-27632 was used. For ICM isolation the whole blastocyst culture method was employed. Currently, this hESC line has been successfully transferred to a feeder-free conditions using matrigel and hMSC-conditioned media. Gra-3 has been maintained feeder-free for 13 passages.

Otras observaciones o información relevantes (a rellenar por el BNLC):
Other comments or relevant information (to be completed by BNLC)

Seguimiento de la línea (a rellenar por el BNLC):
Follow up of the line (to be completed by BNLC)

SECCIÓN 4 Declaración

Confirmo que la información contenida en estos impresos es cierta y asumo total responsabilidad sobre la misma.
I confirm that the information contained in this form is true and I assume total responsibility for it.

Firma en Representación del Centro / Signature in Representation of the Centre *(Representante legal del Departamento/Centro)* *(Legal Reprentative of the Department/Centre)* JUAN JESÚS BANDERA Fecha/ Date:	Firma del Investigador Principal *Signature of the Principal Investigator* PABLO MENENDEZ BUJAN/JOSE LUIS CORTES ROMERO Fecha /Date
Nombre y Cargo de la Persona Representante del Centro: *Name and Position of the Person Representing the Centre:* JUAN JESUS BANDERA. DIRECTOR GERENTE FUNDACIÓN PROGRESO Y SALUD	
Dirección Postal: *Postal Address:* Avda. Americo Vespucio, 5., bloque 2, 2ª planta, 41092, isla de la Cartuja, Sevilla	Teléfono /*Telephone*: +34 955 04 04 50 Fax: +34 955 04 04 57 E-mail: fundacion@fundacionprogresoysalud.org

JUNTA DE ANDALUCIA

CONSEJERÍA DE SALUD
Dirección General de Calidad, Investigación y Gestión del Conocimiento

SALIDA
JUNTA DE ANDALUCIA
CONSEJERIA DE SALUD
24 ABR. 2008
Registro General
11008
37

Banco Andaluz de Células Madre
Centro de Investigación Biomédica
Parque Tecnológico de Ciencias de la Salud
A/A.: Fernando Cobo Martínez
Avda. del Conocimiento, s/n
18100 Armilla (Granada)

ATO/RCG/CRY/rsj S- 1205
Sevilla, 21 de abril de 2008
Comunicando informe favorable proyecto investigación

Por la presente le comunico que reunida el pasado 16 de abril de 2008 la Comisión de Seguimiento y Control de la Donación y Utilización de Células y Tejidos Humanos, acordó INFORMAR FAVORABLEMENTE la ampliación del proyecto titulado **"Optimización de condiciones de cultivo sin "feeders" para líneas de células madre embrionarias humanas (CMEH) importadas o derivadas a partir de embriones donados en fase de pre-implantación: diferenciación de CMEH hacia línea hematopoyética mediante la expresión de Hoxa9 y/o cocultivo con células madre mesenquimales de cordón umbilical"** del que usted es investigador principal. Por lo que el Comité de Investigación con Preembriones Humanos resuelve **AUTORIZAR** la utilización de los 225 preembriones solicitados y aceptados previamente por este Comité.

Atentamente,

EL PRESIDENTE DEL COMITÉ DE INVESTIGACIÓN
CON PREEMBRIONES HUMANOS

JUNTA DE ANDALUCIA
CONSEJERIA DE SALUD
SEVILLA

Fdo. Antonio Torres Olivera

JUNTA DE ANDALUCIA

CONSEJERÍA DE SALUD
Dirección General de Calidad, Investigación y Gestión del Conocimiento

SALIDA
JUNTA DE ANDALUCIA
CONSEJERIA DE SALUD
18 DIC. 2007
Registro General 36.452 Sevilla

Juan Jesús Bandera González
(Director Gerente de Fundación Progreso y Salud)
Avda. Américo Vespucio 5, bq 2-2ª planta. Isla de la Cartuja
41092 SEVILLA

80/07 SAAC

17 de diciembre de 2007

Rdo/ Resolución

Adjunto se remite Resolución de esta Dirección General de Calidad, Investigación y Gestión del Conocimiento, relativa a la autorización de centro de investigación con preembriones sobrantes de las técnicas de fecundación in vitro a Banco Andaluz de Células Madre en sus instalaciones sito en Avda. del Conocimiento s/n de Armilla (Granada).

Asimismo como se indica en la mencionada Resolución, contra la misma, que no pone fin a la vía administrativa, podrá interponerse recurso de alzada, ante el Ilmo. Sr. Secretario de Calidad y Modernización en el plazo de un mes desde su notificación, de conformidad con lo establecido en los artículos 114 y 115 de la Ley 30/92 (en la redacción dada por la Ley 4/99 de 13 de enero), de Régimen Jurídico de las Administraciones y del Procedimiento Administrativo Común.

EL JEFE DE SERVICIO DE AUTORIZACIÓN Y ACREDITACIÓN DE CENTROS,

Fdo. Rufino Domínguez Morales

RECEPCION
JUNTA DE ANDALUCIA
CONSEJERIA DE SALUD
Fundación Progreso y Salud
26/12/07
Registro General 2891 Sevilla
Hora

Fecha: 14/12/2007
N/ Ref: 80/07 SAAC

Examinado el expediente reseñado, instruido a instancias de la Fundación Progreso y Salud, se consideran los siguientes

HECHOS

PRIMERO.- Con fecha 12 de noviembre de 2007, se presentó en el Registro de la Consejería de Salud, solicitud de autorización del Banco Andaluz de Células Madre como centro de investigación con preembriones sobrantes de las técnicas de fecundación in Vitro, conforme a lo previsto en el artículo 13 del Decreto 364/2003, de 22 de diciembre, por el que se regula la organización, composición y funcionamiento del Comité de Investigación con Preembriones Humanos y el procedimiento de autorización de los proyectos y centros de investigación con preembriones sobrantes de las técnicas de fecundación in vitro.

SEGUNDO.- Con fecha 10 de diciembre de2007 se gira visita de inspección a las instalaciones del Banco de Células Madre sito en el Parque Tecnológico de Ciencias de la Salud de Granada, por parte de los técnicos competentes designados a tal fin por esta Dirección General, emitiéndose informe positivo con fecha 12 de diciembre de 2007.

FUNDAMENTOS DE DERECHO

PRIMERO.- Esta Dirección General es competente para resolver este expediente, de conformidad con la regla de competencias establecida en el artículo 13.1 del Decreto 364/2003, de 22 de diciembre, por el que se regula la organización, composición y funcionamiento del Comité de Investigación con Preembriones Humanos y el procedimiento de autorización de los proyectos y centros de investigación con preembriones sobrantes de las técnicas de fecundación in vitro, a tenor de la Disposición Adicional Primera, punto 2, del Decreto 241/2.004, de 18 de mayo, por el que se establece la estructura básica de la Consejería de Salud, y del Servicio Andaluz de Salud.

SEGUNDO.- De conformidad con el artículo 12.2 del citado del Decreto 364/2003, de 22 de diciembre, los centros de investigación que se autoricen deberán cumplir con los siguientes requisitos:

a) Contar con personal con la formación y experiencia necesaria para realizar este tipo de investigaciones.

b) Disponer de procedimientos de trabajo normalizados para la manipulación y procesado de las muestras biológicas.

c) Contar con las instalaciones y los recursos materiales necesarios para la manipulación, el almacenamiento y la conservación de las líneas celulares.

d) Mantener un registro para garantizar una trazabilidad adecuada de las líneas celulares, cuyo acceso deberá estar restringido a las personas autorizadas por el investigador principal, que deberán cumplir las exigencias de confidencialidad establecida en la normativa vigente."

TERCERO.- En la tramitación del expediente se ha cumplido lo dispuesto en el Decreto 364/2003, de 22 de diciembre, la Ley 7/2003 de 20 de octubre, por la que se regula la investigación en Andalucía con preembriones, habiéndose efectuado visita por parte de los técnicos designados por esta Dirección General, y resultando de todo ello informe favorable, conforme al articulo 13.3 de dicho Decreto, por lo que, vistos los preceptos citados y demás de general aplicación, esta Dirección General

RESUELVE

Conceder la autorización de centro de investigación con preembriones sobrantes de las técnicas de fecundación in vitro al Banco Andaluz de Células Madre sito en el Parque Tecnológico de Ciencias de la Salud de Granada.

Contra la presente Resolución, que no pone fin a la via administrativa, podrá interponerse recurso de alzada, ante la Secretaría General de Calidad y Modernización, en el plazo de un mes desde su notificación, de conformidad con lo establecido en los artículos 114 y 115 de la Ley 30/1992 (en la redacción dada por la Ley 4/1999, de 13 de enero), de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común.

Sevilla, 17 de diciembre de 2007

EL DIRECTOR GENERAL DE CALIDAD, INVESTIGACIÓN Y GESTIÓN DEL CONOCIMIENTO

Fdo. Antonio Torres Olivera

CURRICULUM VITAE
February 1st, 2009

PERSONAL INFORMATION:

NAME: Pablo MENENDEZ (November 16th, 1974)
CURRENT POSITION: Director of Andalusian Stem Cell Bank (BACM).
Tenure Team Leader, ISCIII, Madrid, Spain.
CONTACT INFO: Phone: 00 34 958 894671; E-mail: pablo.menendez@juntadeandalucia.es

EDUCATION & TRAINING:

1992-1997	B. Sc. (Biochemistry)	University of Salamanca. Spain.
1997-2002	Ph.D. (Medicine)	University of Salamanca. Spain.
2002-2005	Post-doctoral fellow in Dr. Mick Bhatia's Lab. Stem Cell Biology Program, Robarts Research Institute, University of Western Ontario, London, ON, Canada.	
2005-2007	Jose Carreras Trust Fellow. Section of Hemato-Oncology headed by Prof. Mel Greaves. Institute of Cancer Research, London, UK.	
January 2007-Present	Scientific Director BACM, Granada, Spain	

AWARDS, FELLOWSHIPS AND PROFESSIONAL APPOINTMENTS:

1998	DAKO studentship
1998-2002	Graduate Ph.D. scholarship, Health Ministry of Spain (FIS scholarship).
2002-2004	Robarts postdoctoral fellowship.
2003	International Stem Cell Conference Travel Award. Singapore.October 2003.
2004-2007	Canadian Institutes of Health Research (CIHR) postdoctoral fellowship Funding Reference Number: MFE-68579.
2005	Ramón y Cajal Programme Investigator (Spanish Ministry of Science and Technology). Area of Medicine.
2006	sonal Award "International Foundation Jose Carreras /E.D.Thomas". Reference Number FIJC-05/EDTHOMAS.
2006	Tenure Principal Investigator. Area of Regenerative Medicine and Cell Therapy. Instituto de Salud Carlos III. Madrid. Spain.
2007	entific Director of the Andalusian Stem Cell Bank, Deputy Director of Spanish National Stem Cell Bank.
2008	"Tercer Milenio" Award to under 35 Young Outstanding Investigator. Promoted and funded by The Innovation, Science and Enterprise Andalusian Government.

SCIENTIFIC GRANTS (2004-2008): 900.000 Euros from Autonomic, National and International Funding Bodies

CAPITAL MONEY (2005-2008): 727.000 Euros from ISCIII Infrastructure Calls

PUBLICATIONS:

International peer-review publications: 49 (including Nature, Cell Stem Cell, Nat Methods, Immunity, JEM, Blood, MCB, Leukemia, Stem Cells, Mol Therapy, Plos,…)

National invited publications: 5
Book Chapter: 3

INVITED LECTURES

National: 21
International: 11

PATENTS: 3 (stem cell biology)

SCIENTIFIC ADVISORY BOARDS AND INTERNATIONAL INITIATIVES:

Title: Methods in Molecular Biology- Invited Editor
Institution: Springer Publishing Group
Subject: Stem Cell Banking
Date: 2009

Title: The OPEN Stem Cell Journal
Institution: Bentham Editoral Publishing
Subject: Member of the Editorial Board
Date: October 2008-present

Title: Spanish Scientific Committee for evaluation of stem cell projects
Institution: ISCIII, Subdirección General of Cell Therapy and Regenerative Medicine. Spain
Subject: Stem Cells and Cell Therapy
Date: October 2006

Title: Scientific Committee for evaluation of stem cell-related projects
Institution: Fundación Progreso y Salud. Consejería de Sanidad. Andalucía. Spain
Subject: Stem Cells and Cell Therapy
Date: November 2006

Title: Member of the Scientific Advisory Board of the EU hESC registry
Institution: European Union hESC Registry
Date: March 2007-present

DOCTORAL THESIS: 2 ongoing PhD projects.

CURRICULUM VITAE

February 1st, 2009

PERSONAL INFORMATION:

NAME:	Jose Luis CORTES (December 18th, 1975)
CURRENT POSITION:	Researcher Andalusian Stem Cell Bank (BACM)
CONTACT INFORMATION:	Phone: +34 958 894 672; e-mail: josel.cortes.sspa@juntadeandalucia.es

EDUCATION & TRAINING:

1993-1998	B.Sc. (Biology) University of Granada. Spain
1999	Fellow in Assisted Reproduction Clinic Gutenberg, Malaga, Spain.
1999-2004	Fellow in Hospital Virgen del Rocio (IVF Unit), Seville, Spain
2004-2005	FIS Fellow in Hospital Virgen de las Nieves (Pathology Unit), Granada, Spain.
March 2005-Present	Researcher BACM, Granada, Spain

FELLOWSHIPS:

1999	"Salud y Comunicacion" Foundation Fellowship.
1999-2003	"Promedic" Foundation Fellowship.
2004	"Reina Mercedes" Foundation Fellowship.
2004-2005	"Fondo de Investigación Sanitaria" Fellowship (Spanish Ministry of Health) Reference Number 03/0310.

PUBLICATIONS:

International peer-review publications: 15
National peer-review publications: 4
Book chapter: 1

INVITED LECTURES:

International: 1
National: 6

PATENTS: 1 (stem cell biology)

ANEXO 1

Servicio Andaluz de Salud
CONSEJERÍA DE SALUD

Servicio de Análisis Clínicos (Laboratorio HLA)

IDENTIFICACION	GRA-1
APELLIDOS	
NOMBRE	
H_CLINICA	
DOCTOR	
PROCEDENCIA	EMBRYOLOGIST ANDALUSIAN STEM CELL BANK
FECHA	24-Noviembre-2008
HLA CLASE_I	HLA-A*2402, *3301; B* 0702, *4403; Cw*0220, *0702
HLA CLASE_II	HLA-DRB1*1101, *1102; DQB1*0301, *0301.

Fdo. Dr. Lopez Nevot FEA de Inmunologia
Laboratorio HLA Genómico
Hospital Virgen de las Nieves
Granada

Fdo. Dr. Prof. Federico Garrido
Jefe de Servicio de Análisis Clínicos
Hospital Virgen de las Nieves
Granada

C.S. 7/1

Hospital Universitario Virgen de las Nieves
Avda. Fuerzas Armadas, 2. 18014 - Granada
Tel.: 958 02 00 00. Fax: 958 02 01 32

ANEXO 1

Servicio Andaluz de Salud
CONSEJERÍA DE SALUD

Resultados del análisis de microsatélites de la línea GRA-1

GRA-1	STRs	Allele 1	Allele 2
	D6S311	220.33	248.93
	D6S291	198.32	205.82
	D6S273	122.77	131.48
	c.1.2.5	180.07	196.75
	c.1.2.c	247.26	250.14
	D6S265	122.87	140.65
	D6S105	118.69	122.47
	D6S276	194.86	217.25
	D15S146	217.08	222.71
	D15S1028	178.1	181.83
	D15S126	199.14	204.96
	D15S209	196.17	199.9
	D15S153	214.21	216.12

Granada 24 de noviembre de 2008

Fdo Dr. Prof. Federico Garrido
Jefe de Servicio de Análisis Clínicos
Hospital Virgen de las Nieves
Granada

Fdo Dra. Isabel Maleno
Investigadora FIBAO
Hospital Virgen de las Nieves
Granada

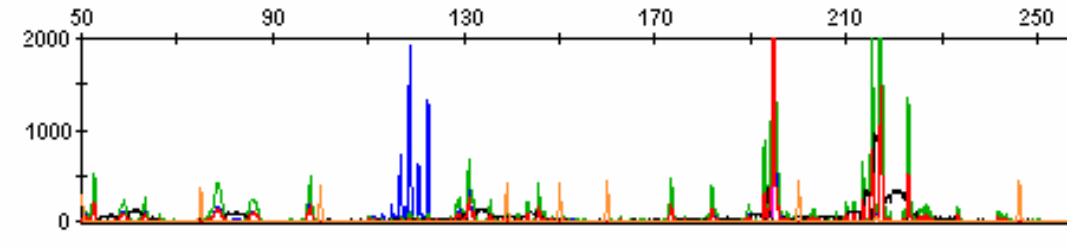
Hospital Universitario Virgen de las Nieves
Avda. Fuerzas Armadas, 2. 18014 - Granada
Tel.: 958 02 00 00. Fax: 958 02 01 32

C.S. 7/1

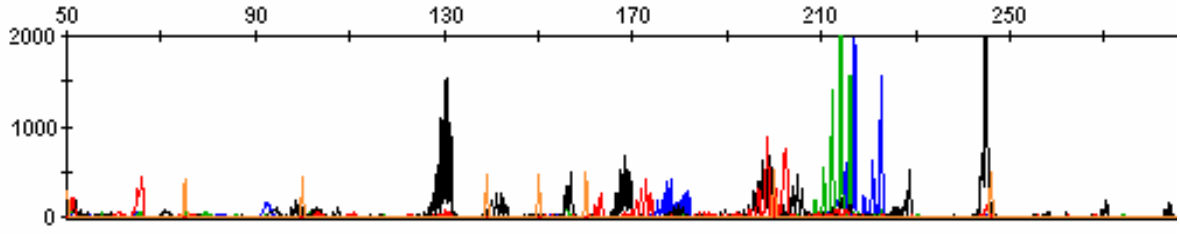
ANEXO 1. CONTINUACION

(LINEA GRA-1)

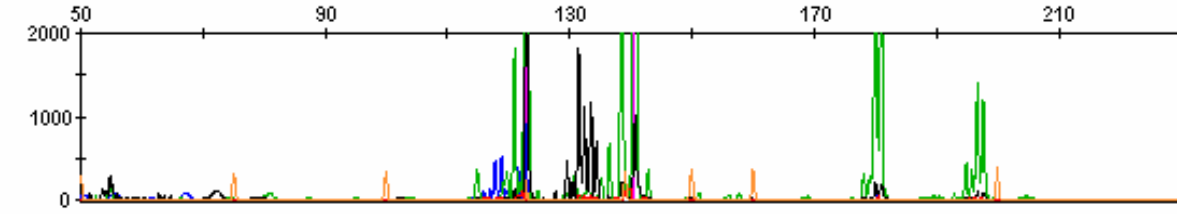
MIX1: **D6S276 + D6S311 + D6S291 + C.1.2.C**



D15S153 + D15S126 + D15S209 + D15S1028 + D15S146



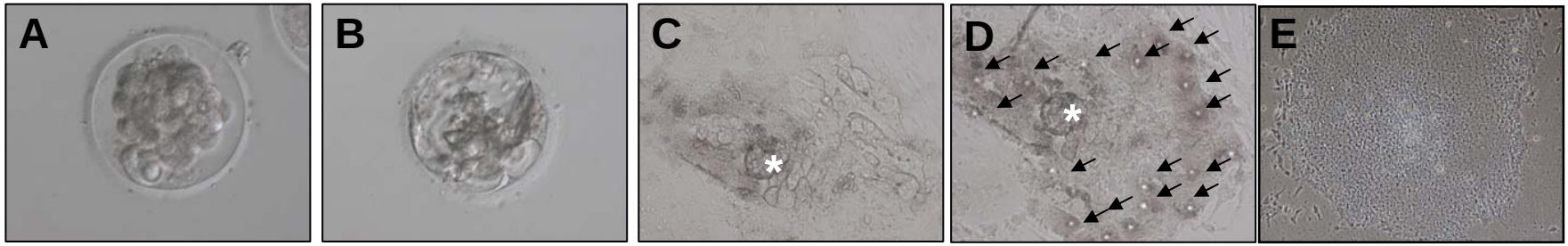
MIX.2 : **D6S105 + D6S265 + D6S273 + C.1.2.C**



Anexo 2:

Secuencia de imágenes de la evolución del embrión descongelado hasta colonia primaria.

Representative images of the thawed embryo throughout the embryonic outgrowth development



GRA-1

ANEXO 3

Servicio Andaluz de Salud
CONSEJERÍA DE SALUD

Unidad de Producción Celular
Hospital Virgen de las Nieves

CULTIVO MICOLÓGICO

MUESTRA: GRA-01 PASE 11. Células madre embrionarias humanas

FECHA DE CULTIVO: 6 de agosto de 2008

TÉCNICA: Cultivo en Agar Sabouraud

RESULTADO: Negativo a los 15 días. No se observa crecimiento de hongos

Granada, 21 de agosto de 2008

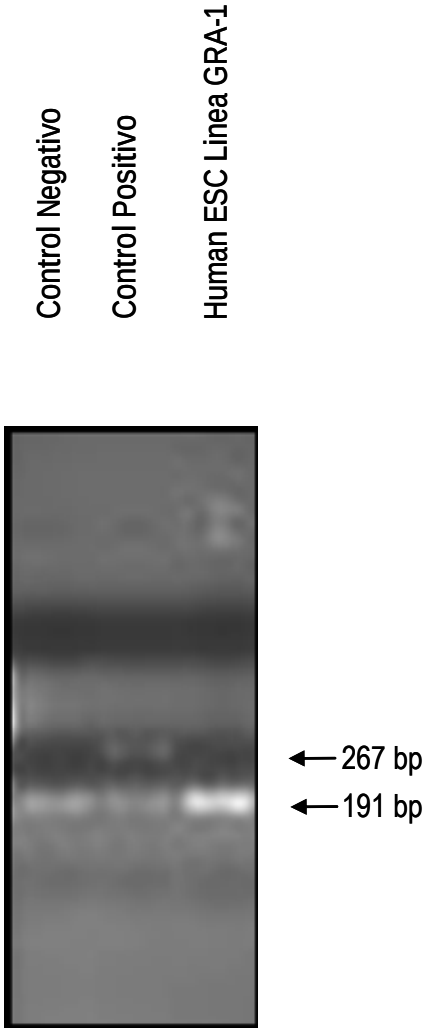
Sara Sanbonmatsu Gámez
Responsable de Calidad
Unidad de Producción Celular
HU Virgen de las Nieves

Hospital Universitario Virgen de las Nieves
Avda. Fuerzas Armadas, 2. 18014 Granada
Teléf.: 958 02 00 00 / 02 01 32

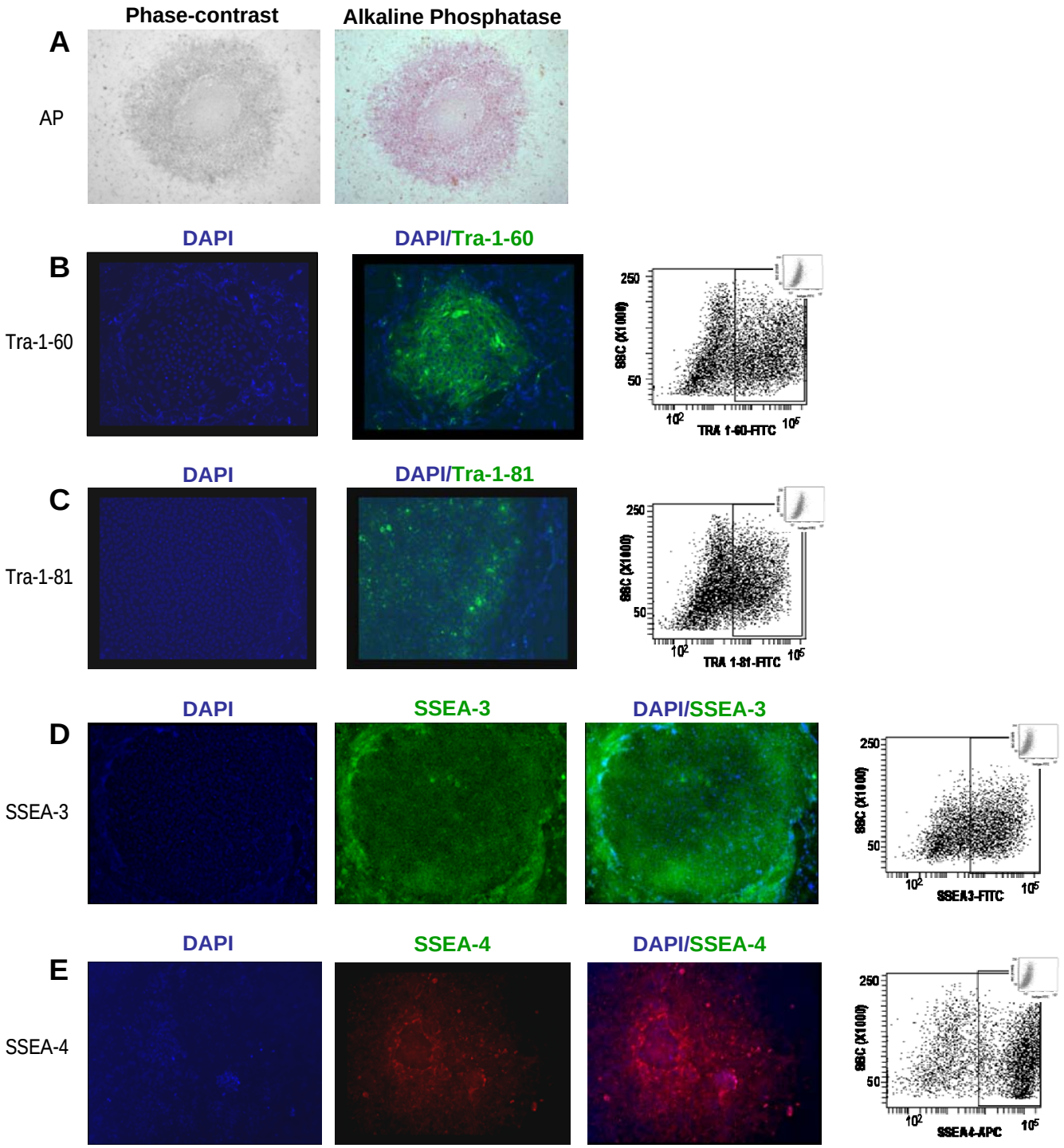
ANEXO 4

Test Microbiológico para Detección de Micoplasma

Línea de hESCs: GRA-1
Fecha: 1/10/2008
Pase: 14 (feeders)
Kit de Detección: Venor Gem (Casa Comercial): Minerva biolabs
Resultado: Negativo
Control +: fragmentos de DNA del genoma de Micoplasma oral
Control - : H_2O

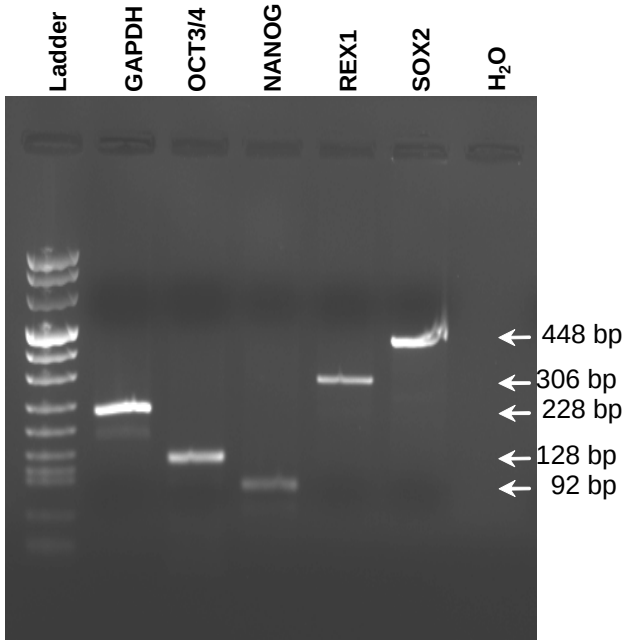


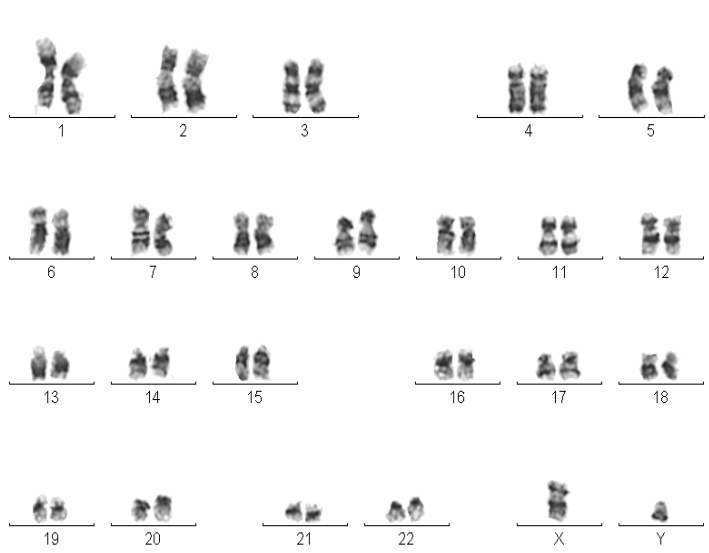
Anexo 5. Línea GRA-1
Caracterización Inmunofenotípica



ANEXO 6. Línea GRA-1

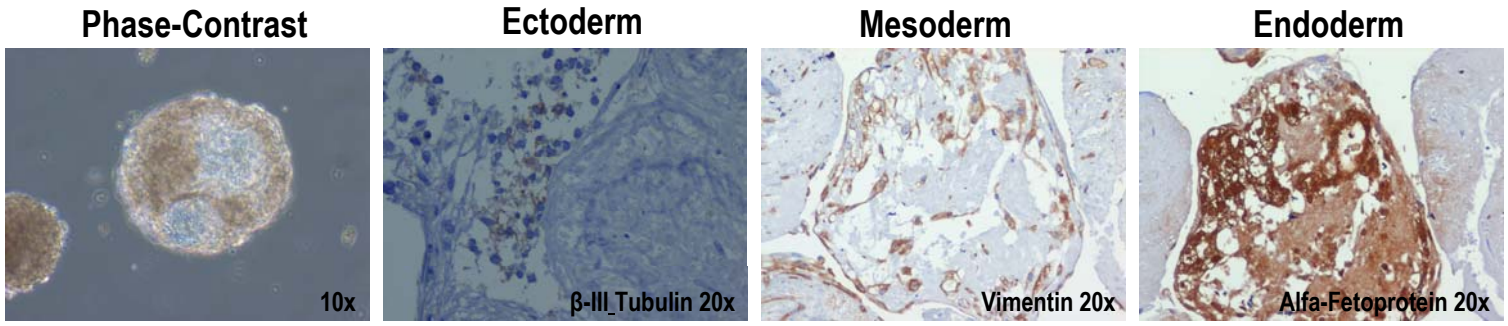
Expresión de Factores de transcripción y cariotipo



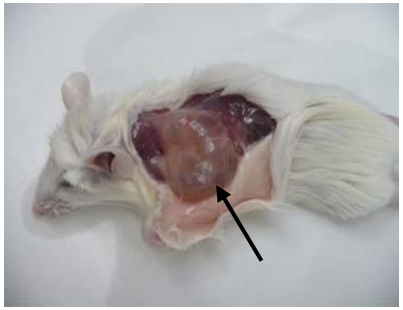


Anexo 7. Línea GRA-1

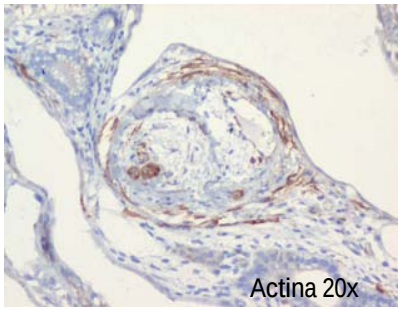
In vitro differentiation (EB formation) of GRA-1 showing tissues representing the three germ layers



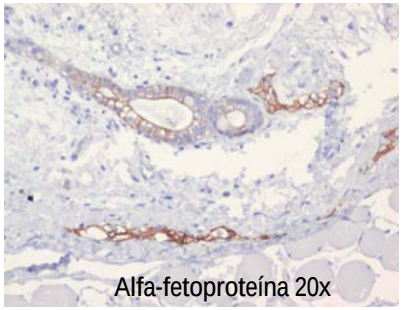
ANEXO 8. Línea GRA-1
(Diferenciación In Vivo-Teratomas)

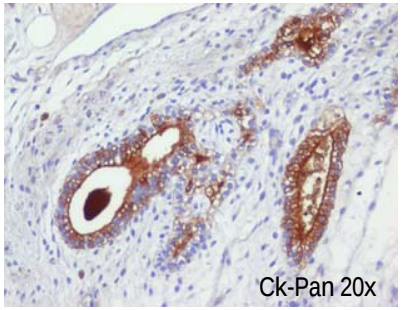


MESO:

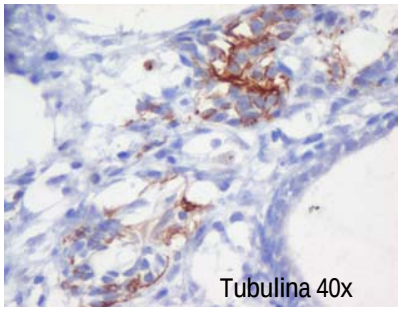


ENDO:





ECTO:



ANEXO 9

Draft Manuscript For Review. Reviewers should submit their review at
http://mc.manuscriptcentral.com/humrep

Mesenchymal stem cells facilitate the derivation of human embryonic stem cells from cryopreserved poor-quality embryos

Journal:	*Human Reproduction*
Manuscript ID:	draft
Manuscript Type:	Original Articles
Date Submitted by the Author:	
Complete List of Authors:	Cortes, Jose; Spanish Stem Cell Bank, Stem Cell Derivation Sanches, Laura; Spanish Stem Cell Bank, Stem Cell Derivation Ligero, Gertrudis; Spanish Stem Cell Bank, Stem Cell Derivation Gutierrez, Ivan; Spanish Stem Cell Bank, Stem Cell Derivation Catalina, Purificacion; Spanish Stem Cell Bank, Stem Cell Derivation Elosua, Carolina; Spanish Stem Cell Bank, Stem Cell Derivation Leone, Paola; Spanish Stem Cell Bank, Stem Cell Derivation Montes, Rosa; Spanish Stem Cell Bank, Stem Cell Derivation Bueno, Clara; Spanish Stem Cell Bank, Stem Cell Derivation Menendez, Pablo; Spanish Stem Cell Bank, Stem Cell Derivation
Keywords:	Mesenchymal Stem Cells, Rock Inhibitor, EMBRYO QUALITY, STEM CELLS
Specialty:	Embryology

scholarONE™
Manuscript Central

ANEXO 9

Mesenchymal stem cells facilitate the derivation of human embryonic stem cells from cryopreserved poor-quality embryos

J.L. Cortes[1]*, L. Sanchez[1], G. Ligero[1], I. Gutierrez-Aranda[1], P. Catalina[1], C. Elosua[1], P.E.Leone[1], R. Montes[1], C. Bueno[1], V. Ramos-Mejía[1], I. Maleno[2], J.L. García-Pérez[1] & P. Menendez[1]*

[1] Andalusian Stem Cell Bank. Centro de Investigación Biomédica. CSJA-UGR. Parque Tecnológico de la Salud, Avda del Conocimiento s/n, Granada, Spain. [2] Servicio de Análisis Clínicos. Hospital Virgen de las Nieves. Granada. Spain.

***Correspondence should be addressed to:**
Pablo Menendez Ph.D
Jose Luis Cortés
Andalusian Stem Cell Bank (BACM).
Centro de Investigación Biomédica
Parque Tecnológico de la Salud
Avda del Conocimiento s/n. Granada, Spain
Phone: 00 34 958 894672
Fax: 00 34 958 894652
Email: pablo.menendez@juntadeandalucia.es ; josel.cortes.sspa@juntadeandalucia.es

Running title: Derivation of hESCs in MSCs.

Key words: hESC derivation, hMSC, ROCK inhibitor, Y-27632, laser drilling, poor-quality frozen embryos.

ANEXO 9

ABSTRACT

Background: Human ESCs have opened up a new area of research in biomedicine. The efficiency of hESC derivation from frozen poor-quality embryos is very low and is normally achieved by plating the embryos on mouse (MEFs) or human foreskin (HFFs) feeders. Here, we attempted to optimize the hESC derivation process from frozen poor-quality embryos. **Methods and Results:** Three conditions were compared for improving embryo survival and hESC derivation efficiency: i) embryo treatment with the ROCK inhibitor Y-27632; ii) the use of human mesenchymal stem cells (hMSCs) as feeders; and iii) the laser drilling (LD) technology for ICM isolation. Two hundred and nineteen frozen poor-quality embryos were used. Thawed embryos were randomly treated with (n=110) or without (n=109) Y-27632. Surviving embryos that developed to blastocyst stage (n=50) were randomly co-cultured on HFFs (n=21) or hMSCs (n=29). The technique used for ICM isolation was either whole-blastocyst culture (WBC) or WBC assisted by LD. Embryo survival experienced a 52% increase upon treatment with Y-27632. Human MSCs facilitated ICM outgrowth and hESC derivation: three hESC lines could be derived on hMSCs (10.3% efficiency) whereas no hESC line could be derived on HFFs (0% efficiency). However, neither ROCK inhibition nor the ICM isolation method improved the efficiency of hESC establishment. The hESC lines derived on hMSCs, termed GRA-1,-2, -3 were fully characterized and showed typical hESC morphology, euploidy, expression of ESC-associated surface markers and transcription factors, and displayed *in vitro* and *in vivo* multilineage developmental potential. These hESC lines have now been maintained stable for over 28 passages on hMSCs and have also been successfully transferred to feeder-free culture conditions and maintained in hMSC-conditioned media. **Conclusion:** Our data suggest that Y-27632 increases embryo survival and that hMSCs significantly facilitate the efficiency of hESC derivation from frozen poor-quality embryos.

ANEXO 9

INTRODUCTION

Human embryonic stem cell (hESC) research represents a nascent area of investigation. Human ESCs are pluripotent stem cells derived from the inner cell mass (ICM) of human blastocyst-stage embryos (Thomson et al.,1998; Reubinoff et al., 2000). They are defined by both robust self-renewal capacity and pluripotent developmental potential *in vitro* and *in vivo* (Menendez et al., 2006). Accordingly, cultures of hESCs represent a theoretically inexhaustible source of pluripotent cells that can be differentiated into any cell type and therefore, have been hailed as a unique tool for a range of biomedical applications such as cell replacement therapy, developmental biology, drug discovery and disease modelling (Bueno et al., 2008a; Menendez et al., 2005).

The blastocyst quality, the ICM isolation method used and the culture conditions (feeders) employed are likely the three main factors dictating either ESC derivation failure or success (Cortes et al., 2007a, 2008). Regarding the blastocyst quality, in many countries worldwide only cryopreserved human embryos created during *in vitro* fertilization (IVF) which are in excess of clinical need or deemed clinically useless based on poor morphology may be used for hESC derivation (Cortes et., 2007b). These frozen supernumerary human embryos are usually of poor-quality (Lerou et al., 2008). The current methods to isolate the ICM are still controversial. The ICM is usually isolated from the expanded blastocysts using a variety of techniques, including immunosurgery (Solter et al., 1975), mechanical processes (Bongso et al.,1994), and whole blastocyst culture (WBC) methods (Kim et al., 2005). These ICM isolation methods are associated with some challenges, including the use of xeno-components, which may prevent the use of hES cell derivatives in potential future therapeutic applications, and the low ES cell establishment efficiency (Cortes et al., 2008). Recently, we and others have developed a laser-assisted system for the isolation of the ICM from mouse and human embryos (Tanaka et al., 2006; Cortes et al., 2007a, 2008; Turetsky et al., 2008). In addition, the derivation and maintenance of hESCs normally

ANEXO 9

require the use of feeder cells (Amit et al., 2003). Up until now, either MEFs, STO fibroblast (Park et al., 2004) or HFFs (Hovatta et al., 2003) were used as supporting feeders for hESC line derivation. Despite that MEFs and HFFs are the most commonly used feeders for hESC co-culture, we and others have recently showed successful hESC maintenance in human mesenchymal stem cells (hMSCs) or hMSC-conditioned media (Cheng et al., 2003; Montes et al., 2009). In line with the mesoderm origin of hMSCs, we hypothesize that hESC lines derived on hMSCs may be more prone to differentiation toward mesodermal lineages (Ledran et al., 2008). Thus, the optimization of hESC line derivation methods is challenging and it is still unclear which approach is the most efficient (Hovatta et al., 2006).

Human ESCs are susceptible to apoptosis upon cell detachment and dissociation (Watanabe et al., 2007). The selective Rho-associated kinase (ROCK) inhibitor Y-27632 has been reported to increase the survival of dissociated hESCs and their cloning efficiency and to improve their survival upon crypreservation (Watanabe et al., 2007; Martin-Ibañez et al., 2008; Li et al., 2008, 2009). However, whether treatment of cryopreserved human embryos with the ROCK inhibitor Y-27632 facilitates embryo survival and augments the efficiency of hESC establishment still needs to be examined.

In the present study, we attempted to optimize the hESC derivation process from frozen poor-quality embryos. Three experimental conditions were compared for improving embryo survival and hESC derivation efficiency: i) embryo treatment with the selective ROCK inhibitor Y-27632; ii) the use of hMSCs as feeders and, iii) the laser drilling (LD) technology for ICM isolation. Our data suggest that hMSCs significantly increase the efficiency of hESC derivation from frozen poor-quality embryos. In contrast, despite treatment with Y-27632 inhibitor increased embryo survival, neither ROCK inhibition nor the ICM isolation method used seems to enhance the efficiency of hESC establishment.

ANEXO 9

MATERIALS AND METHODS

Cryopreserved Human Embryos

This study was approved by our Local Authorities and the Spanish National Embryo Steering Committee. Cryopreserved human embryos (n=219) were donated to this study upon informed consent by couples that had already undergone an IVF cycle (Cortes et al., 2007b). These human embryos were frozen between 1995 and 2004.

As shown in our experimental design **(Figure 1)**, the embryos were initially thawed in the presence or absence of 10 μM of ROCK inhibitor Y-27632 (Sigma, St Louis, MO) (Narumiya et al., 2000), which has been previously reported to increase the survival of single clones of hESCs, their cloning efficiency, their survival upon crypreservation (Watanabe et al., 2007; Martin-Ibañez et al., 2008; Li et al., 2008, 2009). Y-27632 inhibitor was added to the human embryo culture media, G-1 v.5 and G-2 v.5 (Vitrolife, Sweden).

Embryo outgrowth co-culture on allogeneic human feeders

Human embryos which survived and developed up to the blastocyst stage were cultured in tissue treated four-well plates (BD Labware, Franklin Lakes, NJ) either on a confluent layer of γ-ray-inactivated (4000 rads) HFFs (Hovatta et al., 2003) or hMSCs (Schaffler and Buchler, 2007; Ucelli et al, 2008; Garcia-Castro et al., 2008). HFFs were purchased from ATCC (SCD-1112SK). Human MSCs were obtained from post-natal adipose tissue from healthy donors upon informed consent as previously described (Cobo et al., 2008; Garcia-Castro et al., 2008). HFFs and hMSCs were grown in IMDM and advanced-DMEM, respectively, supplemented with 10% FCS and 2mM L-glutamine. The four-well plates were seeded with $0.5x10^5$ cells/cm^2 HFFs or hMSCs. Successful embryo outgrowths were routinely maintained in medium consisting of 80% KO-DMEM supplemented with

ANEXO 9

20% KO Serum Replacement, 1% non-essential amino acids, 1mM L-glutamine, 0.1 mM β-mercaptoethanol and 8ng/mL of bFGF (all from Invitrogen, CA), independently of the allogeneic human feeders.

ICM Isolation Methods

Two different experimental approaches were used for ICM isolation and further hESC line establishment depending on the quality of the blastocyst: WBC method alone or WBC assisted by laser (WBC+LD) (Cortes et al., 2008). This latter ICM isolation method has been previously described (Cortes et al., 2008). WBC assisted by LD was employed for blastocysts with a large and distinguishable ICM, while WBC alone was used for those blastocysts with a tiny or indistinguishable ICM (Kim et al., 2005; Cortes et al., 2007a, 2008). Briefly, when WBC was used, the zona pellucida was removed by Tyrode´s Acid (Irvine Scientific, CA, USA) for no more than one minute. Then, the whole-blastocyst was cultured in such a way that the trophoectoderm cells and the ICM cells adhered to the feeders **(Figure 2A-D)**. Subsequently, the distinguishable ICM was carefully plucked and transferred to a freshly prepared human feeder layer and allowed to expand.

Those blastocysts treated with WBC+LD were initially treated with Tyrode´s Acid for no more than one minute, to assure the complete dissolution of zona pellucida, in such a way that the trophoectoderm cells and the ICM adhered to the feeder layer. By day 3 of culture the trophoectoderm cells began to expand leaving the ICM accessible, forming a dome-like structure (Cortes et al., 2008). At this point, the trophectoderm cells were laser-shot, leaving the ICM free of trophoectoderm cells and reducing the risk of dragging the trophectoderm cells when the ICM was plucked and transferred to a freshly prepared human feeder layer (Cortes et al., 2008) **(Figure 2A-E)**.

http://humrep.oupjournals.org

ANEXO 9

Characterization of established hESCs

Established hESCs were characterized by indirect immunocytochemistry using antibodies against SSEA-3, SSEA-4 (Developmental Studies Hybridoma Bank, University of Iowa, USA), TRA-1-60 and TRA-1-81 (Chemicon, CA). Briefly, hESC colonies were cultured in chamber slides. Cells were fixed in 4% of paraformaldehyde for 20 minutes followed by 30 minutes incubation in 10% normal goat serum in PBS. Colonies were incubated with primary antibodies (1:100 dilution in PBS) for 1 hour at room temperature (RT). A FITC-conjugated anti-mouse IgM secondary antibody (1:100 dilution in PBS) was used for 30 minutes at RT (Jackson Laboratories Inc). The slides were mounted in Vectashield containing DAPI (Vector Laboratories Inc). As a negative control, the primary antibodies were replaced by PBS. The same markers were assessed by flow cytometry. For flow cytometry analysis, an irrelevant isotype-match was used. Tripsin-dissociated hESC lines were suspended in PBS+3%FBS at a concentration of 2-5x10^4 cells per 100 µL and incubated with the specific primary antibody for 30 minutes at 4º C. After washing with PBS+3%FBS, cells were incubated with 2.5 µL of FITC-conjugated goat anti-mouse IgG antibody (Immunotech, Marseille, France). After 15 minutes at RT, the cells were washed in PBS+3%FBS and finally stained with 7-aminoactinomycin D (7-AAD) (Immunotech) for 5 minutes at RT. Live cells identified by 7-AAD exclusion were analyzed for expression of SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 using a FACSCanto-II flow cytometer equipped with the FACSDiva software (BDB) (Bueno et al., 2008). Alkaline phosphatase expression was demonstrated using the AP Detection Kit (Chemicon).

Molecular and cytogenetic analysis

The expression of the pluripotency-associated transcription factors Oct3/4, Nanog, Rex-1 and Sox2 was assessed by end-point RT-PCR (Catalina et al., 2008a). GAPDH was used as a housekeeping gene control. The following PCR conditions were used: 5 min at 94ºC, 35 cycles of 30 seconds at

ANEXO 9

94ºC followed by 50 seconds at 60ºC and 50 seconds at 72ºC and a final extension of 10 min at 72ºC and products were resolved in 1% agarose gel (Catalina et al., 2008a)

Conventional karyotyping analysis was performed as previously described in detail and twenty to thirty metaphase spreads per hESC line were analyzed (Menendez et al., 2005; Catalina et al., 2008a, 2008b).

In vitro **differentiation analysis**

Near confluent hESCs were treated with collagenase IV for 5 min at 37ºC, transferred ($2x10^2$ cells/cm^2) to non-adherent plates and allowed to differentiate spontaneously by embryoid body (EB) formation in DMEM supplemented with 20% FBS, 1% L-glutamine, 0.1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol with media changes every 4 days. After 21 days of EB differentiation, EBs were spun down, fixed with 4% paraformaldehyde for 10 minutes and embedded in paraffin (Catalina et al., 2008a). For each staining, three sections per specimen were used. Then, the cells were incubated (1 hour at RT) with the primary antibodies anti-α-fetoprotein (Santa Cruz Biotechnology; 1:500 dilution in PBS), anti-β-III Tubulin (Chemicon, 1:100 dilution in PBS) and anti-smooth-muscle actin (Chemicon, 1:100 dilution in PBS). Slides were then incubated with a byotinylated secondary antibody (30 minutes at RT) and a streptavidin peroxidase complex (30 minutes at RT) (both from Vector Laboratories Inc). The immunostaining was visualized using diaminobenzidine and counterstained with hematoxylin. All the washing steps were done in PBS.

In vivo **teratoma formation into NOD/SCID-IL2Rγ$^{-/-}$ mice**

ANEXO 9

During routine passage, 20-50 clumps consisting of about 100 undifferentiated cells each were harvested and injected into the testis of 6 to 8-week-old NOD/SCIDIL2Rγ$^{-/-}$ mice. Eight to ten weeks later, the resulting teratomas were fixed in 10% neutral buffered formalin, embedded in paraffin, and examined histologically after hematoxylin and eosin staining as previously described (Cortes et al., 2008; Catalina et al., 2008a).

Short Tandem Repeats (STR) Typing

STR analysis was carried out using the Geneprint® Fluorescent STR Multiplex- 290 GammaSTR® kit (Promega, Madison, WI). Electropherogram data were collected with 292 the ABI PRISM 3100 DNA Sequencer (Applied Biosystems), and analyzed using the 293 Genotyper®3.7 software (Applied Biosystems) as previously described (Catalina et al., 2008a).

RESULTS

Y-27632 Rock inhibitor increases cryopreserved embryo survival

Y-27632 has been reported to increase the survival of dissociated hESCs and their cloning efficiency and to improve their survival upon cryopreservation (Watanabe et al., 2007; Martin-Ibañez et al., 2008; Li et al., 2008, 2009). However, whether the treatment of cryopreserved human embryos with Y-27632 facilitates embryo survival still needs to be examined. As shown in our experimental approach (**Figure 1**), 219 poor-quality human embryos which had been cryopreserved between 1995 and 2004 were thawed to establish hESC lines. Embryos were randomly treated with (n=110) or without (n=109) 10μM of the Y-27632. Treatment with Y-27632 induced a 52% increase in embryo survival: 30 surviving embryos out of 110 thawed embryos

(27.3%) and 20 surviving embryos out of 109 thawed embryos (18%) in the presence and absence of Y-27632, respectively. Thus, Y-27632 treatment increases the survival of thawed embryos.

Human MSCs facilitate the derivation of hESC lines from frozen poor-quality embryos regardless the ICM isolation method

Next, we attempted to optimize the hESC derivation process from frozen poor-quality embryos treated with or without Y-27632. We compared the effect of two distinct human feeders (hMSCs *versus* HFFs) and two different ICM isolation methods on the efficiency of hESC establishment. Surviving embryos which reached the blastocyst stage were randomly transferred to a feeder layer composed of hMSCs or HFFs to ascertain which of these allogenic human feeders better support the expansion of the ICM and subsequent establishment of hESC lines. The ICM isolation method consisted of whole blastocyst culture (WBC) alone for blastocysts with indistinguishable ICM or WBC followed by laser drilling (LD) for human blastocysts with a large and distinguishable ICM.

Out of the fifty embryos which survived the cryopreservation procedure and reached the blastocyst stage (**Figure 2A-C**), 29 were plated on hMSCs and 21 on HFFs (**Figure 1**). The ICM was subsequently isolated using either WBC (n=39, **Figure 2D**) or laser-assisted WBC (n=11, **Figure 2E**), depending on the blastocyst quality. ICM outgrowths (**Figure 2F**) were allowed to expand. Expanded ICMs were subsequently transferred to freshly prepared feeders and allowed to grow further to give rise to hESC lines. Human MSCs used as feeders facilitated ICM outgrowth and hESC derivation: three hESC lines could be derived on hMSCs (3 hESC lines out of 29 blastocysts; 10.3% efficiency) whereas no hESC line could be derived on HFFs (0 hESC lines out of 21 blastocysts; 0% efficiency) (**Figure 1**). However, neither ROCK inhibition nor the ICM isolation method contributed to an increased efficiency of hESC establishment (**Figure 1**). The three hESC lines derived on hMSCs termed GRA-1,-2, -3 were fully characterized. The STR typing

ANEXO 9

profile **(Supplementary Figure 1)** clearly differs among GRA-1, GRA-2 and GRA-3 confirming the unique identity of these independently derived hESC lines and lack of cross-contamination among them. Taken together, our data suggest that hMSCs significantly increase the efficiency of hESC derivation from frozen poor-quality embryos while neither ROCK inhibition nor the ICM isolation method seems to enhance the efficiency of hESC establishment.

***In vitro* and *in vivo* full characterization of the hESC lines derived on hMSCs**

GRA-1, GRA-2 and GRA-3 hESC lines have been cultured for over 28 passages so far in either hMSCs and also in feeder-free conditions using matrigel and MSC-conditioned media (MSC-CM). No differences in *in vitro* and *in vivo* pluripotency and culture homeostasis were observed among these hESC lines maintained in hMSCs and those grown in MSC-CM (data not shown). These hESC lines derived on hMSCs retained typical hESC morphology **(Figure 2G,H)** and expression of the pluripotency-associated surface markers AP **(Figure 3A)**, Tra-1-60 **(Figure 3B)**,Tra-1-81 **(Figure 3C)**, SSEA-3 **(Figure 3D)** and SSEA-4 **(Figure 3E)**. Immunocytochemistry staining was confirmed by flow cytometry **(Figure 3 right panels)**. Similarly, all the three hESC lines expressed the transcription factors Oct3/4, Nanog, Rex-1 and Sox-2 **(Figure 4A)** and remained karyotypically stable **(Figure 4B)**. Functionally, these hESC lines successfully differentiated *in vitro* through EB formation **(Figure 5A; left panel)** into tissues representing the three germ layers: ectoderm **(**β-III-Tubulin+ cells; **Figure 5A)**, mesoderm **(**Actin+ cells; **Figure 5A)** and endoderm **(**α-fetoprotein+ cells; **Figure 5A)**. The gold-standard pluripotency assay relies on the ability to form teratomas *in vivo* upon injection into immune-deficient mice. The three hESC lines derived and maintained in hMSCs formed teratomas 8-10 weeks after inoculation. These complex and disorganized tumours

ANEXO 9

contained a variety of tissues representing the three germ layers **(Figure 5B)**, demonstrating the pluripotent features of the hESC lines derived on hMSCs.

DISCUSSION

This study has prospectively examined the use of the Rock inhibitor Y-27632 and hMSC feeders on embryo survival and hESC derivation efficiency from a large cohort of poor-quality human embryos which had been cryopreserved for 4 to 13 years. In addition, two distinct ICM isolation methods (WBC alone *versus* WBC combined with laser-assisted technology) were compared side by side in this cohort of human embryos.

Treatment of cryopreserved embryos with Y-27632 induced a 52% increase in embryo survival. Y-27632 inhibitor has been recently reported to improve the survival of dissociated and cryopreserved hESCs growing in suspension (Watanabe et al., 2007; Martin-Ibañez et al., 2008; Li et al., 2008, 2009). Li *et al* (Li et al., 2009) have very recently proposed through elegant studies a potential mechanism of action for Y-27632. Y-27632 seems to augment survival of hESCs not only by decreasing the level of apoptosis but also through complementary mechanisms such as increasing cellular adhesion by promoting stronger cell-cell interaction between cells (Li et al., 2009). However, to the best of our knowledge, no study has been performed to determine whether Y-27632 inhibitor also improves survival of cryopreserved human embryos. The 52% increase in embryo survival we report, opens up new avenues for investigators and clinicians not only in the field of hESC research but also in human assisted reproduction to explore further potential applications of Y-27632 or analogous compounds in a variety of clinical scenarios. From a more clinical standpoint, whether Y-27632 facilitates IVF, embryo development toward blastocyst stage, improves the efficiency of fertilized eggs achieving the blastocyst stage, enhances embryo survival upon cryopreservation are still unanswered questions. We envision that the previously reported

ANEXO 9

data about the role of Y-27632 on hESCs and our data showing the role of Y-27632 in enhancing human embryo survival upon cryopreservation will encourage further investigations aimed at optimizing protocols in IVF clinics.

We also demonstrate for the first time that hMSCs significantly increase the efficiency of hESC derivation from frozen poor-quality embryos as compared to HFFs (10.5% *versus* 0%). Three hESC lines (GRA-1, GRA-2 and GRA-3) were successfully derived in hMSCs and have now been maintained genetically stable for over 28 passages on hMSCs and have also been successfully transferred to feeder-free culture and maintained in hMSC-CM. They have been fully characterized and show typical hESC morphology, euploidy, expression of ESC-associated surface markers and transcription factors, and displayed *in vitro* and *in vivo* multilineage developmental potential. The optimization of hESC derivation methods is advisable because all human embryos are a limited resource and, in many countries only cryopreserved human embryos (normally of poor-quality) are legally permitted for research (Cortes et al., 2007b, 2008b). Interestingly, neither the freeze-thaw technique, treatment with Y-27632 nor the ICM isolation method influenced the hESC derivation efficiency. We previously reported with fresh mouse embryos that the efficiency of mESC derivation is superior when the whole-blastocyst culture is combined with laser-assisted technology (Cortes et al., 2008a). However, the laser drilling technology did not offer any improvement over the whole-blastocyst culture when frozen poor-quality human embryos were used instead of fresh mouse embryos which are of better quality. The cellular and molecular mechanisms underlying the role of hMSCs in facilitating embryo development and hESC establishment needs to be elucidated. The signalling pathways that regulate embryo development and promote ESC establishment are largely unknown. However, key developmental pathways including Sonic hedgehog, Notch, Wnt, and bone morphogenic proteins are potential candidates (Bailey et al., 2007; Bueno et al., 2007). In fact, a number of soluble factors produced by supportive

ANEXO 9

stroma/mesenchymal stem cells with novel stem cell expansion activities have been recently identified as part of pathways associated with mesodermal induction (Hutton et al., 2007). It should be mentioned that hESC lines have been successfully derived in HFFs using fresh embryos (Hovatta et al., 2006; Inzunza et al 2003). However, to the best of our knowledge this is the first prospective study comparing two distinct human feeders to improve the derivation of hESC lines from frozen poor-quality embryos.

Culture-expanded hMSCs largely support human hematopoietic stem cells in long-term assays and their differentiation into myeloid, erythroid, megakaryocytic, osteoblastic or B-cell lineages, even in the absence of added cytokines (Cheng et al., 2003). In line with the mesoderm origin of hMSCs, we hypothesize that hESC lines derived on hMSCs may be more prone to differentiation toward mesodermal lineages (Ledran et al., 2008). In fact, preliminary data show that beating hESC colonies and differentiating EBs are much more frequently observed during routine culture in comparison with other hESC lines derived on non-hMSC feeders. Studies are ongoing in our Lab to determine and quantify to what extend these hESC lines derived on hMSCs are predisposed to differentiation into multiple mesodermal tissues.

ACKNOWLEDGMENTS

This work was funded by the Health Department (0028/2006 & 0030/2006 to P.M) and Innovation Department (P08-CTS-03678 to P.M) of Andalusian Government, The Spanish Ministry of Health (FIS PI070026 to PM and FIS PI08171 to J.L.G-P). Research in J.L.G-P´s Laboratory is also supported by the ISCIII-CSJA (EMER07/056) and Marie Curie IRG (FP7-PEOPLE-2007-4-3-IRG). C.B is supported by the FIS-ISCIII (CP07/00059) and the Jose Carreras International Foundation against the Leukemia (FIJC-05/EDThomas 2006). V.R-M is supported by The Marie Curie (FP7-PEOPLE-IIF-2008). We are indebted to ANACER (Spanish Association of IVF clinics) and Dr.

ANEXO 9

Santiago Alvarez (University of Extremadura) for provision of cryopreserved human embryos from couples who voluntary signed an informed consent. We also thank Teresa de la Cueva, Dr. Sara Sanbonmatsu, Dr. Gema Lucena, Ruth Rubio, Dr. Javier García-Castro and Prof. Francisco Nogales for their outstanding technical assistance.

REFERENCES

Amit M, Margulets V, Segev H, Shakiri K, Laevski I, Coleman R and Itskovitz-Eldor J (2003) Human feeder layers for human embryonic stem cells. Biol Reprod 68, 2150-2156.

Bailey JM, Singh PK, Hollingsworth MA (2007) Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. J Cell Biochem,102:829-839.

Bongso A, Fong CY, Ng SC, Ratnam S (1994) Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 9,2110-2117.

Bueno C, Lopes LF, Menendez P (2007) Bone marrow stromal cell-derived Wnt signals as a potential underlying mechanism for cyclin D1 deregulation in multiple myeloma lacking t(11;14)(q13;q32). Blood Cells Mol Dis, 39:366-368.

Bueno C, Montes R, Garcia-Castro J, Greaves M, Menendez P (2008) Human embryonic stem cells: A potential system for modelling infant leukemia harbouring MLL-AF4 fusion gene. Drug Discov Today: Dis Models 4, 53-60.

Bueno C, Montes R, Martin L, Prat I, Hernandez MC, Orfao A, Menendez P (2008) NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursors: is NG2 expression in leucemia dependent on the target cell where leukemogenesis is triggered?. Leukemia 22, 1475-1478.

Catalina P, Bueno C, Montes R, Nieto A, Ligero G, Sanchez L, Jara M, Rasillo A, Orfao A, Cigudosa J, et al. (2008) Genetic stability of human embryonic stem cells: a first-step toward the

ANEXO 9

development of potencial hESC-based systems for modeling childhood leukaemia. Leuk Res. DOI: 10.1016/j.leukres.2008.08.028.

Catalina P, Montes R, Ligero G, Sanchez L, de la Cueva T, Bueno C, Leone PE, Menendez P (2008) Human ESC predisposition to karyotipic instability: Is a matter of cell culture adaptation or differential vulnerability among hESC lines due to inherent properties?. Mol Cancer 7; 76-82.

Cheng L, Hammond H, Ye Z, Zhan X, Dravid G (2003) Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells 21:131-142.

Cobo F, Navarro JM, Herrera I, Vivo A, Porcel D, Hernández C, Jurado M, García-Castro J, Menendez P (2008) Electron microscopy reveals the presence of viruses in MEFs but neither in HFFs or hMSCs used for hESC maintenance: toward an implementation of microbiological quality assurance program in stem cell banks. Clonning and Stem Cells 10, 65-74 .

Cortes JL, Cobo F, Catalina P, Nieto A, Cabrera C, Montes R, Concha A and Menendez P (2007) Evaluation of the laser technique method to isolate the inner cell mass of murine blastocysts. Biotechnol Appl Biochem 46, 205-209.

Cortes JL, Antiñolo G, Martinez L, Cobo F, Barnie AH, Zapata A and Menendez P (2007) Spanish stem cell bank interviews examine the interest of couples in donating surplus human IVF embryos for stem cell research. Cell Stem Cell 1, 17-20.

Cortes JL, Sánchez L, Catalina P, Cobo F, Bueno C, Martínez-Ramirez A, Barroso A, Cabrera C, Ligero G, Montes R et al. (2008) Whole-blastocyst culture followed by laser drilling technology enhances the efficiency of inner cell mass isolation and embryonic stem cell derivation from good- and poor-quality mouse embryos: new insights for derivation of human embryonic stem cell lines. Stem Cells Dev 17, 255-267.

ANEXO 9

Cortes JL, Menendez P (2008) Reproductive medicine meets human embryonic stem cell (hESC) research: the need to adjust the regulatory framework to actual expectations and potential detrimental consequences of hESC research. Fertil Steril. 10.1016/j.fertnstert.2008.05.041.

Garcia-Castro J, Trigueros C, Madrenas J, Perez-Simon JA, Rodriguez R, Menendez P (2008) Mesenchymal stem cells and their use as cell replacement therapy and disease modeling tool. J Cell Mol Med 12, 1-14.

Hovatta O, Mikkola M, Gertow K, Strömberg AM, Inzunza J, Hreinsson J, Rozell B, Blennow E, Andäng M and Ahrlund-Richter L (2003) A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 18, 1404-1409.

Hovatta O (2006). Derivation of human embryonic stem cells: towards clinical quality. Reprod Fertil Dev 16: 823-828.

Hutton JF, D'Andrea RJ, Lewis ID (2007) Potential for clinical ex vivo expansion of cord blood haemopoietic stem cells using non-haemopoietic factor supplements. Curr Stem Cell Res Ther, 2:229-37.

Inzunza J, Gertow K, Strömberg MA, Matilainen E, Blennow E, Skottman H, Wolbank S, Ahrlund-Richter L, Hovatta O (2005) Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells, 23: 544-549.

Kim HS, Oh SK, Park YB, Anh HJ, Sung KC, Kang MJ, Lee LA, Suh CS, Kim SH, Kim DW et al. (2005) Methods for derivation of human embryonic stem cells. Stem Cells 23:1228-1233.

Ledran MH, Krassowska A, Armstrong L, Dimmick I, Renström J, Lang R, Yung S, Santibanez-Coref M, Dzierzak E, Stojkovic M et al. (2008) Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches Cell Stem Cell 3, 85-98.

ANEXO 9

Lerou PH, Yabuuchi A, Huo H, Takeuchi A, Shea J, Cimini T, Ince TA, Ginsburg E, Racowsky C and Daley GQ (2008) Human embryonic stem cell derivation from poor-quality embryos Nat Biotechnol 26, 212-214.

Li X, Meng G, Krawetz R, Liu S and Rancourt DE (2008) The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. Stem Cells Dev 17, 1079-1085.

Li X, Krawetz R, Liu S, Meng G and Rancourt DE (2009) ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. Hum Reprod (in press).

Martin-Ibañez R, Unger C, Strömberg A, Baker D, Canals JM and Hovatta O (2008) Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor. Hum Reprod 23, 2744-2754.

Menendez P, Wang L and Bhatia M (2005) Genetic manipulation of human embryonic stem cells: a system to study early human development and potential therapeutic applications. Current Gene Therapy 5, 375-385.

Menendez P, Bueno C, Wang L (2006). Human embryonic stem cells: A journey beyond cell replacement therapies. Cytotherapy 8, 530-541.

Montes R, Ligero G, Sanchez L, Catalina P, de la Cueva T, Nieto A, Melen G, Rubio R, García-Castro J, Bueno C, Menendez P (2009) Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-β and IGF-II. Cell Res (in press).

Narumiya S, Ishizaki T and Uehata M (2000) Use and properties of ROCK-specific inhibitor Y-27632. Methods Enzymol 325, 273-284.

ANEXO 9

Park SP, Lee KS, Ah Shin H, Cho HY, Chung KS, Kim EY and Lim JH (2004) Establishment of human embryonic stem cell lines from frozen-thawed blastocysts using STO cell feeder layers. Hum Reprod 19, 676-684.

Reubinoff BE, Pera MF, Fong CY, Trounson A and Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399-404.

Schäffler A and Büchler C (2007) Concise review: adipose tissue-derived stromal cells-basic and clinical implications for novel cell-based therapies. Stem Cells 25, 818-827.

Solter D and Knowles BB (1975) Immunosurgery of mouse blastocysts. Proc Natl Acad Sci USA 72, 5099-5102.

Tanaka N, Takeuchi T, Neri QV, Sills ES and Palermo GD (2006) Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model. J Transl Med 4, 20-32.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshal VS and Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147.

Turetsky T, Aizenman E, Gil Y, Weinberg N, Shufaro Y, Revel A, Laufer N, Simon A, Abeliovich D, Reubinoff BE (2008) Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. Hum Reprod 3:46-53.

Uccelli A, Moretta L and Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8, 726-736.

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25,681-686.

ANEXO 9

LEGEND TO FIGURES:

Figure 1. Experimental strategy used for the derivation of hESC lines from poor-quality frozen embryos using hMSCs. Two hundred and nineteen frozen poor-quality human embryos were used in the present study. Embryos were randomly treated with (n=110) or without (n=109) a chemical ROCK inhibitor (Y-27632). Surviving embryos which reached the blastocyst stage were transferred to a feeder layer of hMSCs or HFFs to ascertain which of these allogenic human feeders better support the expansion of the ICM and subsequent establishment of hESC lines. The ICM isolation method consisted of whole blastocyst culture (WBC) followed by laser drilling (LD) for human blastocysts with a large and distinguishable ICM. For blastocysts with very small or indistinguishable ICM, WBC was used for ICM isolation. Established hESC lines were fully characterized by morphology, immunocytochemistry, flow cytometry, karyotyping, STR analysis and *in vitro* and *in vivo* differentiation potential into the three germ layers.

Figure 2. Representative pictures depicting the hESC derivation process from frozen-thawed human embryos. A) Representative day-3 poor-quality embryos immediately after thawing. **B)** Blastocyst in the cavitation stage. **C)** Poor-quality expanded blastocyst. **D)** Outgrowth of a representative blastocyst adhered to hMSCs 3 days after ICM isolation (WBC technique). The asterisk indicates the ICM. **E)** Outgrowth of a representative blastocyst adhered to hMSCs 3 days after isolation method (WBC+LD). The asterisk shows the ICM. The black arrows depict the exact laser shots (white dots). **F)** Replating of the expanded ICM onto hMSCs on passage 1 of culture. **G)** Typical morphology of a hESC colony growing on hMSCs after >30 passages. **H)** A T25-flask phase-contrast image showing multiple hESC colonies growing on MSCs for >30 passages.

ANEXO 9

Figure 3. Phenotypic characterization of the hESCs derived on hMSCs. A) Phase-contrast image (left panel) and representative alkaline phosphatase (AP, right panel) staining. **B)** Immunochemistry staining against Tra-1-60. **C)** Immunochemistry stainig against Tra-1-81. **D)** Immunochemistry staining against SSEA-3. **E)** Immunochemistry staining against SSEA-4. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI, blue). The right panels confirm the expression of Tra-1-60, Tra-1-81, SSEA-3 and SSEA-4 by flow cytometry. Isotypes are shown as insets.

Figure 4. Molecular and cytogenetic characterization of the hESCs derived on hMSCs. A) RT-PCR analysis showing expression of Oct 3/4, Nanog, Rex1 and Sox2 in undifferentiated hESC cultures. GAPDH was used as housekeeping gene. **B)** A representative G-banding analysis depicting euploid karyotype.

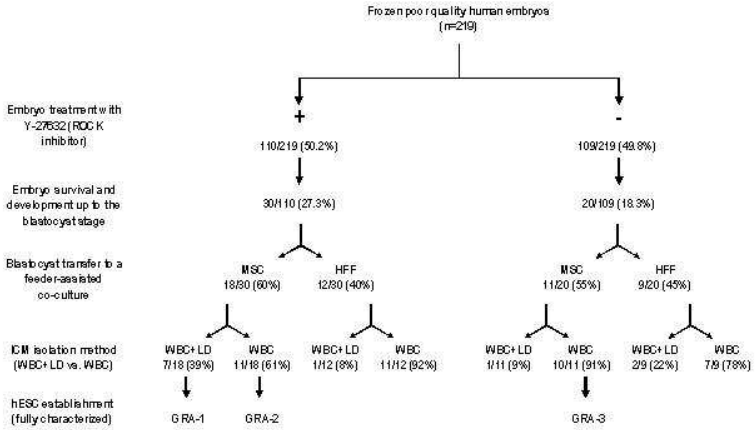
Figure 5. hESC lines derived on hMSCs and maintained in hMSCs or hMSC-CM retain potential for three germ layer differentiation. A) Histological analysis of EBs showing spontaneous *in vitro* differentiation into ectoderm (β-III Tubulin +), mesoderm (Actin +) and endoderm (α-fetoprotein +). **B)** Teratomas developed 6-10 weeks after inoculation of hESCs under the testicular capsule. Histological studies revealed the presence of tissues representing the three germ layers: ectoderm (left panels), mesoderm (middle panels) and endoderm (right panels). Representative image of a control testicle non-injected with hESCs showing normal seminiferous tubules is shown.

Suppl Figure 1. STR Analyses of the newly derived hESC lines. Distinct STR profiles for GRA-1, GRA-2 and GRA-3 hESC lines.

http://humrep.oupjournals.org

ANEXO 9

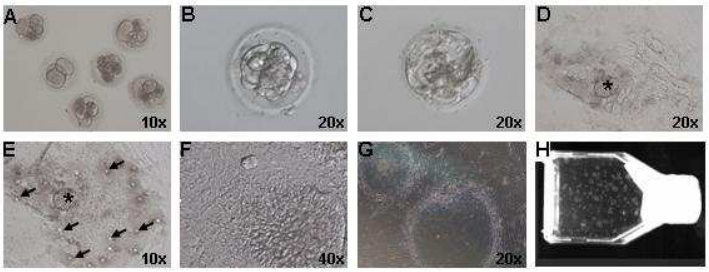
Figure 1. Cortes *et al*



190x254mm (96 x 96 DPI)

ANEXO 9

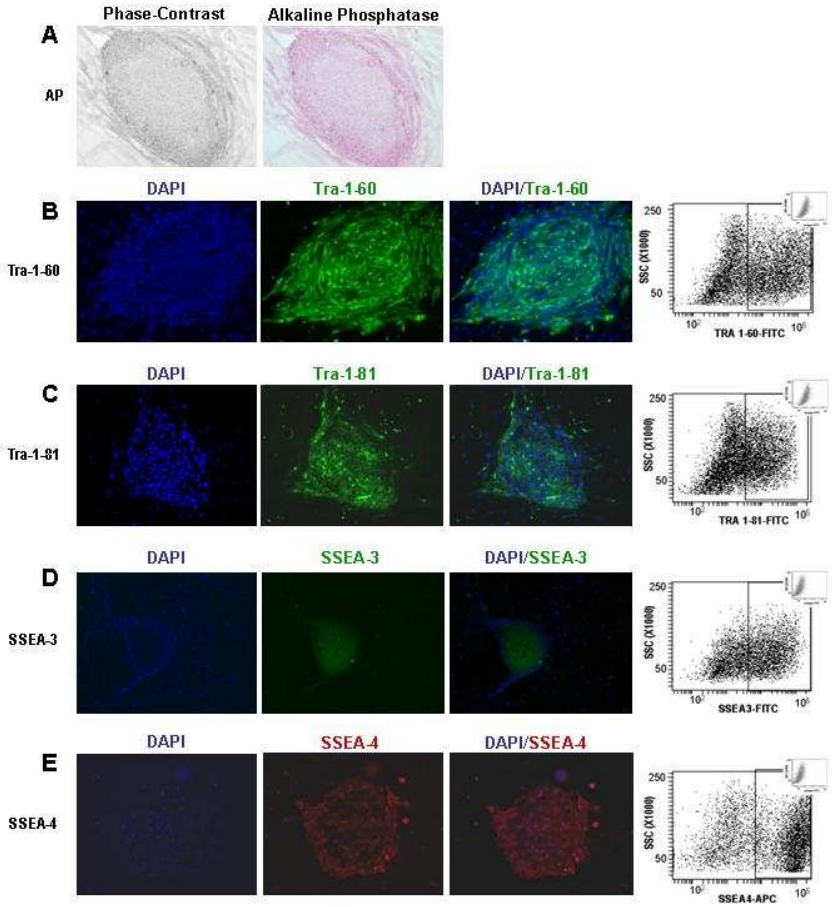
Figure 2. Cortes *et al*



190x254mm (96 x 96 DPI)

ANEXO 9

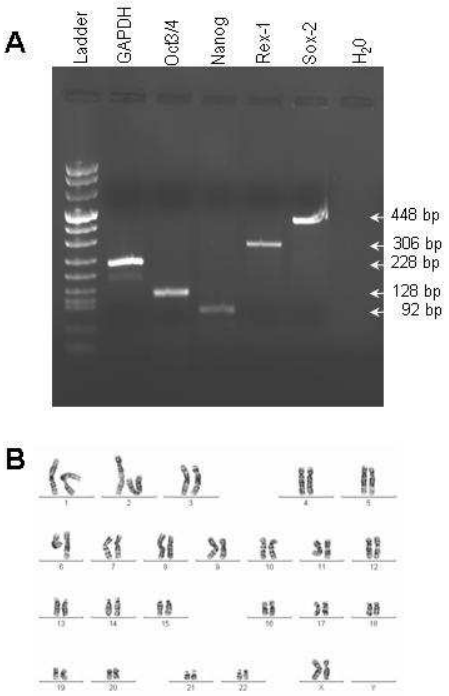
Figure 3. Cortes *et al*



190x254mm (96 x 96 DPI)

ANEXO 9

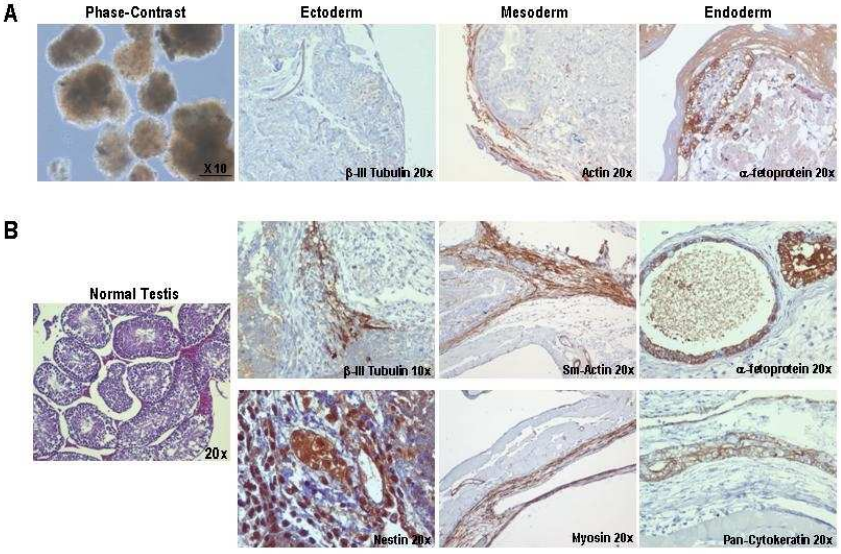
Figure 4. Cortes *et al*



190x254mm (96 x 96 DPI)

ANEXO 9

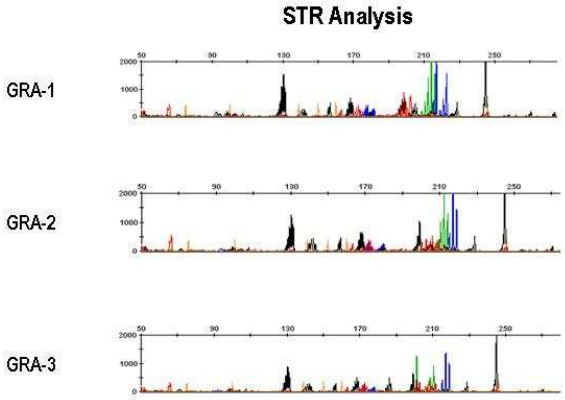
Figure 5. Cortes *et al*



254x190mm (96 x 96 DPI)

ANEXO 9

Suppl Figure 1. Cortes *et al*



254x190mm (96 x 96 DPI)