HEIDI HONGISTO

# Fibroblast Feeder Cells in Human Pluripotent Stem Cell Culture and Retinal Differentiation

## Progress toward clinical cell therapy

ACADEMIC DISSERTATION
To be presented, with the permission of
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Kanslerinrinne 1, Tampere,
on January 25th, 2013, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, Institute of Biomedical Technology
Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB)
Finland

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Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 40 190 9800
taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1796
ISBN 978-951-44-9011-8 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1272
ISBN 978-951-44-9012-5 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2013

*To Fanny and Arto*

# Abstract

The establishment of human pluripotent stem cells (hPSCs) has raised great excitement for their prospective use in the field of regenerative medicine. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) can self-renew indefinitely and generate virtually any differentiated cell type, thus serving as an unlimited cell source for cell replacement therapies. One promising clinical application of hPSC-based cell therapies is the treatment of retinal degenerative diseases such as age-related macular degeneration (AMD), which is the leading cause of blindness among the elderly in the western world. Phase one clinical trial on the treatment of AMD with retinal pigment epithelial (RPE) cells differentiated from hESCs was recently commenced.

The standard *in vitro* culture conditions for undifferentiated hPSCs contain undefined and xenogeneic compounds, exposing the cells to infectious and immunogenic molecules. Although hESCs cultured under such conditions have recently been approved for clinical trials, the use of xenogeneic cell culture material is highly undesirable. Ideally, clinical grade hPSC lines are established using xeno-free culture protocols and according to Good Manufacturing Practice (GMP)-quality requirements. Also, the molecular mechanisms controlling stem cell differentiation to the eye field and further to the RPE fate are poorly understood. Consequently more efficient and defined differentiation protocols are required.

This thesis aimed at testing and optimising xeno-free and GMP-compatible culture protocols for hESCs regarding hESC culture media, feeder-independent culture methods, and feeder cells. Further, the feeder cell contribution to maintenance of pluripotent hESC status and the induction of RPE differentiation were studied.

As a result, human serum (HS) supplemented hESC culture medium was found to suboptimally support xeno-free hESC culture on human feeder cells. Commercial feeder-independent culture methods were found to support long-term hESC culture while feeder-independent and xeno-free methods published by academic research

groups were found to be unsupportive. Xeno-free, defined media as well as matrices based on human extracellular matrix (ECM) proteins are today on the market for both feeder-dependent and feeder-independent hPSC culture. Xeno-free human feeder cells were established and clinical grade culture protocols for the feeder cells were optimized. These processes could in the future also serve in the establishment of clinical grade hiPSC lines from dermal tissue. Human dermal fibroblast (hDF) feeder cells were found unsupportive of hESCs while commercial human foreskin fibroblasts (hFF) supported long-term hESC propagation in xeno-free culture system.

The feeder cells were found to differ significantly in their ECM protein, especially laminin, and corresponding integrin receptor expression. Laminin-511 was found to contribute to hESC maintenance in feeder-dependent culture as laminin-511 was secreted only by hESC supportive feeder cells. Other factors with a possible role in the maintenance of pluripotency were identified. Feeder cell conditioned media were found to enhance RPE differentiation of hESCs and hiPSCs. Higher activin A secretion by mouse embryonic fibroblast (mEF) feeder cells was hypothesized to be an inductive agent and a corresponding level of activin A significantly augmented hESC-RPE differentiation.

The results of this thesis have contributed to the translation of hPSC research to the clinic by developing suitable culture conditions and provided insights into the mechanisms of maintenance of hESC pluripotency and induction of hPSC-RPE differentiation.

# Tiivistelmä

Ihmisen pluripotentit kantasolut mahdollistavat uudenlaisten soluterapiahoitojen kehittämisen regeneratiivisen lääketieteen käyttöön. Ihmisen alkion kantasolut (hESC) ja geneettisesti pluripotenttiin tilaan uudelleen ohjelmoidut indusoidut kantasolut (hiPSC) voivat jakautua erilaistumattomina erittäin pitkiä aikoja ja myös erilaistua kaikiksi aikuisen yksilön solutyypeiksi. Näiden ominaisuuksiensa vuoksi hESC- ja hiPSC-soluista voidaan tuottaa rajattomasti soluja kliinisiin kantasoluterapioihin. Yksi lupaavimmista käyttökohteista on verkkokalvon rappeumasairauksien, kuten silmänpohjan ikärappeuman hoito. Silmänpohjan ikärappeuma on länsimaissa yleisin yli 65-vuotiaiden sokeutumisen syy. Ihmisen alkion kantasoluista erilaistettuja verkkokalvon pigmenttiepiteelisoluja (RPE) testataan parhaillaan silmänpohjan ikärappeuman hoitoon ensimmäisissä kliinisissä kokeissa.

Tällä hetkellä yleisesti käytössä olevat kantasolujen laboratorioviljelyolosuhteet sisältävät tuntemattomia ja eläinperäisiä ainesosia, jotka altistavat kantasolut eläinpatogeeneille ja immunogeenisille eläinperäisille molekyyleille. Vaikka ensimmäisiin kliinisiin kokeisiin käytettävät solut on viljelty käyttäen eläinperäisiä aineita, niiden käyttö ei ole suositeltavaa. Kliiniseen käyttöön tarkoitetut kantasolut tulisi eristää, viljellä ja erilaistaa Good Manufacturing Practise (GMP)-laatustandardiston mukaisesti, ilman eläinperäisiä materiaaleja. Myös kantasolujen erilaistumista verkkokalvon soluiksi ohjaavat tekijät tunnetaan heikosti ja tehokkaampien erilaistusmenetelmien kehittäminen on tärkeää.

Tämän väitöskirjatyön tavoitteena oli testata ja optimoida eläinperäisiä ainesosia sisältämättömiä ja GMP-soveltuvia erilaistumattomien kantasolujen viljelyliuoksia, tukisoluja ja tukisoluttomia viljelymenetelmiä. Lisäksi tavoitteena oli tutkia tukisolujen merkitystä erilaistumattomien kantasolujen viljelyssä ja erilaistamisessa RPE-soluiksi.

Tämän työn tuloksena ihmisen seerumia sisältävän eläinkomponentittoman viljelyliuoksen todettiin osittain ylläpitävän kantasolujen erilaistumatonta kasvua

tukisolujen päällä. Kaupalliset viljelymenetelmät mahdollistivat kantasolujen tukisoluttoman pitkäaikaisviljelyn mutta akateemisten tutkimusryhmien julkaisemat tukisoluttomat ja eläinkomponentittomat viljelymenetelmät eivät tukeneet kantasolujen erilaistumatonta kasvua. Nykyään erilaisia eläinkomponentittomia viljelyliuoksia ja ihmisen soluväliaineita sisältäviä kasvualustoja on markkinoilla kantasolujen viljelyyn tukisoluilla ja ilman tukisoluja. Tutkimuksen tuloksena perustettiin eläinkomponentittomia tukisolulinjoja ja tukisoluille optimoitiin kliiniseen käyttöön soveltuvat viljelymenetelmät. Näitä prosesseja voidaan tulevaisuudessa käyttää myös kliiniseen käyttöön tarkoitettujen hiPSC-solujen tuottamiseen ihon sidekudossoluista. Ihmisen ihokudoksesta eristetyt sidekudossolulinjat eivät soveltuneet kantasolujen tukisoluiksi, kun taas ihmisen esinahan sidekudossolut tukivat kantasoluviljelyä.

Kantasoluja tukevien ja tukemattomien tukisolujen todettiin eroavan soluväliaineen proteiinien, etenkin laminiinien, ja niiden integriinireseptorien tuotossa. Laminiini-511 tunnistettiin kantasolujen kasvua ylläpitäväksi tukisolujen erittämäksi laminiiniksi, sillä vain kantasoluja tukevat tukisolut tuottivat laminiini-511-tyyppiä. Tutkimuksen tuloksena identifioitiin myös uusia mahdollisia kantasolujen kasvua ylläpitäviä tukisolujen tuottamia tekijöitä. Tukisolujen erittämien tekijöiden todettiin myös edistävän kantasolujen erilaistumista RPE-soluiksi, minkä todettiin osittain johtuvan aktiviini A -kasvutekijän erityksestä. Lisäämällä aktiviini A -kasvutekijää viljelyliuokseen, saatiin erilaistumista merkittävästi tehostettua.

Tämän tutkimuksen tulokset ovat osaltaan myötävaikuttaneet kantasolujen kliiniseen käyttöön soveltuvien viljelymenetelmien kehittämiseen ja laajentaneet näkemystä kantasolujen erilaistumattomuuden säätelystä ja erilaistumisesta RPE-soluiksi.

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# List of abbreviations

| | |
|---|---|
| AMD | Age-related macular degeneration |
| ApoB-100 | Apolipoprotein B-100 |
| April | Proliferation-inducing ligand |
| BAFF | B cell-activating factor belonging to tumor necrosis factor receptor and ligand superfamily |
| BCAM | Basal cell adhesion molecule |
| BIO | 6-bromoindirubin-3′-oxime |
| BMP | Bone morphogenetic protein |
| BrdU | 5-Bromo-2'-deoxyuridine |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CDM | Chemically defined medium |
| CHX10 | Homeobox-containing transcription factor 10 |
| CKI | Casein kinase I (Wnt signaling inhibitor) |
| CM | Conditioned medium |
| c-Myc | V-myc myelocytomatosis viral oncogene homolog (avian) |
| CRALBP | Cellular retinaldehyde-binding protein |
| DAPI | 4,6-diamidino-2-phenylindole |
| Dkk1 | Dickkopf-related protein 1 (Wnt antagonist) |
| DMEM | Dulbecco's modified Eagle's medium |
| DMEM/F12 | Dulbecco's modified Eagle's medium: nutrient mixture F-12 |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid (c, complementary) |
| DNMT3B | DNA (cytosine-5-)-methyltransferase 3 beta |
| DPBS | Dulbecco's phosphate buffered saline |
| EB | Embryoid body |
| EC cell | Embryonic carcinoma cell |
| ECM | Extracellular matrix |
| EG cell | Embryonic germ cell |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| EMEA | European Medicines Agency |
| EMT | Epithelial-mesenchymal-transition |
| ERBB | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinases |
| ESC | Embryonic stem cell (h, human) |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FDA | Unites States Food and Drug Administration |
| FGF | Fibroblast growth factor (b, basic; R, receptor) |
| FITC | Fluorescein isothiocyanate isomer 1 |
| Flt3 | Fms-like tyrosine kinase 3 |

| | |
|---|---|
| GABA | Gamma-aminobutyric acid |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GDF | Growth and differentiation factor |
| GMP | Good manufacturing practice (c, current) |
| GTP | Good tissue practice |
| HA | Hyaluronic acid |
| hDF | Human dermal fibroblast |
| hECM mix | Human ECM mixture of collagen IV, vitronectin, fibronectin, and laminin |
| hESC-df | Human embryonic stem cell-derived fibroblast |
| hFF | Human foreskin fibroblast |
| HLA | Human leukocyte antigen |
| HPLC-MS | Liquid chromatography–mass spectrometry |
| HS | Human serum |
| HSA | Human serum albumin |
| HSPG | Heparan sulfate proteoglycans |
| ICM | Inner cell mass |
| ICR | Imprinting Control Region |
| IGF | Insulin-like growth factor (BP, binding protein) |
| IGF1 LR3 | Insulin like Growth Factor-1 Long R3 |
| IMDM | Iscove's modified Dulbecco's medium |
| iPSC | Induced pluripotent stem cell (h, human) |
| ISCI | International Stem Cell Initiative |
| ITS | Insulin, transferrin, selenium |
| IVF | *In vitro* fertilization |
| JAK | Janus kinase |
| KAL1 | Kallmann syndrome 1 sequence |
| KGF | Keratinocyte growth factor |
| Klf-4 | Krupper-like family of transcription factor 4 |
| Ko-DMEM | Knockout™ -Dulbecco’s modified Eagle’s medium |
| ko-SR | Knockout™ Serum Replacement |
| Lefty | Left-right determination factor 1 |
| LIF | Leukemia inhibitor factor |
| Lin-28 | Lin-28 homolog (C. elegans) |
| MCB | Master cell bank |
| mEF | Mouse embryonic fibroblast |
| MERTK | Mer Tyrosine Kinase |
| MET | mesenchymal-to-epithelial transition |
| mhESC | Modified hESC medium |
| MITF | Microphthalmia-associated transcription factor |
| MSC | Mesenchymal stem cell (h, human) |
| Nanog | Nanog homeobox |
| NEAA | Non-essential amino acids |
| Neu5Gc | N-glycoylneuraminic acid |
| NIC | Nicotinamide |
| Nodal | Nodal homolog |
| Oct-3/4 | Octamer-3/4, POU domain, class 5, transcription factor 1 |
| OTX | Orthodenticle homeobox |
| PAX6 | Paired box protein-6 |
| p.c. | Post coitum, after mating |

| | |
|---|---|
| PCR | Polymerase chain reaction (RT, reverse transcription; q-quantitative real-time) |
| PDTEC | Poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) |
| PE | Phycoerythrin |
| PEDF | Pigment epithelium derived factor |
| PFA | Paraformaldehyde |
| PLDLA | Poly-L,D-lactide 96/4 |
| PMEDSAH | Poly[2-(methacryloyloxy)ethyl-dimethyl-(3-sulfopropyl)ammonium hydroxide] |
| PMEL | Premelanosome protein |
| PSC | Pluripotent stem cell (h, human) |
| RA | Retinoic acid |
| RAX | Retina and anterior neural fold homeobox |
| RCS | Royal College of Surgeons |
| RGD | Arginine-Glycine-Aspartic acid |
| RNA | Ribonucleic acid (m, messanger, mi micro) |
| ROCKi | Rho-associated kinase inhibitor |
| RPE (cell) | Retinal pigment epithelium (epithelial cell) |
| RPE65 | Retinal pigment epithelium-specific 65 kDa protein |
| RT | Room temperature |
| SB-431542 | ALK4 inhibitor (TGFß signaling inhibitor) |
| SCID | Severe combined immunodeficiency |
| SCNT | Somatic cell nuclear transfer |
| SIX3 | Homeobox 3 |
| Smad | Transforming growth factor beta ligand |
| SOP | Standard operating procedure |
| Sox-2 | Sex determining region Y-box 2 |
| SSEA | Stage-specific embryonic antigen |
| STAT | Signal Transducers and Activator of Transcription |
| TDGF1 | Teratocarcinoma-derived growth factor 1 (cripto) |
| TER | Transepithelial electric resistance |
| TGF-$\beta$ | Transforming growth factor beta |
| Ti | Titanium |
| $TiO_2$ | Titanium dioxide-coated titanium |
| TRA | Tumor-related antigen |
| UTF-1 | Undifferentiated embryonic cell transcription factor 1 |
| VEGF | Vascular endothelial growth factor |
| WCB | Working cell bank |
| Wnt | Wingless-type MMTV integration site family |
| ZFP-42 | Zinc finger protein 42 homolog (REX-1) |
| ZO-1 | Zona occludens-1 |
| $ZrO_2$ | Zirconium dioxide-coated titanium |

# List of original publications

The present thesis is based on the following Articles, which are referred to in the text by their Roman numerals (I-IV):

I Rajala K, **Hakala H**, Panula S, Aivio S, Pihlajamäki H, Suuronen R, Hovatta O, Skottman H. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod 2007, 22(5):1231-8.[#]

II **Hakala H**, Rajala K, Ojala M, Panula S, Areva S, Kellomäki M, Suuronen R, Skottman H. Comparison of Biomaterials and Extracellular Matrices as a Culture Platform for Multiple, Independently Derived Human Embryonic Stem Cell Lines. Tissue Eng Part A 2009, 15(7):1775-8.

III **Hongisto H**, Vuoristo S, Mikhailova A, Suuronen R, Virtanen I, Otonkoski T, Skottman H. Laminin-511 expression is associated with the functionality of feeder cells in human embryonic stem cell culture. Stem Cell Res 2012, 8:97–108.

IV **Hongisto H***, Mikhailova A*, Hiidenmaa H, Ilmarinen T, Skottman H. Low level of activin A secreted by fibroblast feeder cells accelerates early stage differentiation of retinal pigment epithelial cells from human pluripotent stem cells. Stem Cell Discovery 2012, 2(4):176-186.

[#] Publication has been previously included in the Doctoral Thesis: Development of Human Stem Cell Culture Conditions for Clinical Cell Therapy, Kristiina Rajala, University of Tampere, 2010

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# 1. Introduction

Human pluripotent stem cells (hPSCs) are self-renewing cells capable of nearly unlimited proliferation combined to the capacity to differentiate to all of the over 200 somatic cell types of the adult. Human embryonic stem cells (hESCs) are derived from surplus embryos of *in vitro* fertilization (Thomson et al., 1998), while human induced pluripotent stem cells (hiPSCs) are generated by genetically reprogramming adult somatic cells back to pluripotent state by a set of transcription factors (Takahashi et al., 2007). A variety of functional cell types have been differentiated from hPSCs *in vitro*, including hepatocytes, insulin producing β cells of the pancreas, contracting cardiomyocytes, and various types of neurons and retinal cells (Vazin and Freed, 2010).

Human PSCs serve as a valuable tool for understanding the complex mechanisms involved in the development of specialized cells and in the establishment of organ structures, they model human genetic diseases and are valuable for drug and toxicology studies. Moreover, the indefinite self-renewing ability and plasticity of hPSCs enables the *in vitro* generation of an unlimited number of clinically relevant cell types, and has opened new avenues in regenerative medicine. The greatest therapeutic promise of hPSCs is to generate specialized cells to replace damaged tissue in patients suffering from devastating degenerative diseases. (Vazin and Freed, 2010)

However, there are still many concerns regarding the safety of the clinical use of pluripotent cells. The mechanisms controlling self-renewal, pluripotency and lineage restriction to various cellular phenotypes are not completely understood. Furthermore, for progression of hPSC-based therapies towards clinical applications, appropriate culture conditions must be developed to generate genetically stable homogenous cell populations and to prevent any adverse effects following transplantation. The *in vitro* culture conditions of undifferentiated hPSCs include animal derived, undefined components like mouse embryonic fibroblast (mEF) feeder cells and fetal bovine serum (FBS), which expose the stem cells to the

incorporation of xeno-pathogens and immunogenic molecules (Martin et al., 2005). Also, the *in vitro* differentiation strategies are often undefined and inefficient for producing clean populations of mature, functional cell types. Clinical grade hESCs and their differentiated progeny should be established and cultured using xeno-free, defined materials and under strict Good Manufacturing Practice (GMP)-quality requirements to ensure the consistency and safety of the cell products (Unger et al., 2008b). Many improvements in the hPSC culture protocols have been achieved in recent years as the factors controlling pluripotent stem cell fate have been elucidated. The use of animal sera and reagents has been replaced with human sourced, recombinant or synthetic alternatives. Feeder-independent culture conditions have been introduced and GMP-compatible culture protocols optimized. This study contributes to the testing and optimization of clinically compliant hESC culture protocols and discusses advances made in the field.

One of the most promising targets for hPSC-therapies is the treatment of blinding disorders such as age-related macular degeneration (AMD) with hPSC-derived retinal pigment epithelial (RPE) cells, and the first clinical trials to test the safety of hESC-RPE cells in AMD patients were recently initiated (Schwartz et al., 2012). The hPSC-RPE cell differentiation strategies still largely rely on spontaneous differentiation as cell aggregates or on feeder cells. In this study the mechanism of feeder aided hESC-RPE cell differentiation was studied to facilitate the development of defined and more robust differentiation strategies.

# 2. Review of the literature

## 2.1 Classification and sources of stem cells

Stem cells are a special class of cells characterized by their ability to self-renew *i.e.* to multiply to generate same kind of cells, and produce progenitor cells which are committed to give rise to fully differentiated cells. Stem cells can be classified according to their potency to differentiate or to their origin.

Totipotent cells are able to create an entire organism, with all its differentiated cell types. During early human embryogenesis, the fertilized egg, the zygote, undergoes mitotic cell divisions to generate a structure called the morula. The zygote and its early progeny up to the 8-cell stage of the morula retain their totipotency. Subsequently, a blastocyst consisting of an inner cell mass (ICM) and an outer trophoblast is formed. The cells of the trophoblast form the extra-embryonic tissue while the undifferentiated cells of the ICM develop into the embryo. The cells of the ICM are pluripotent and retain the ability to develop into all cell types of the three embryonic germ layers: endoderm, mesoderm, and ectoderm, and also germ cells. The embryonic stem (ES) cells are derived from these pluripotent cells. Also, the embryonic germ (EG) cells that give rise to male and female gametes, and embryonic carcinoma (EC) cells derived from germ line tumors called teratocarcinomas show pluripotency. Differentiated cells can also be re-differentiated back to the pluripotent state with different methods, most importantly by genetic reprogramming to create induced pluripotent stem (iPS) cells. (Wobus and Boheler, 2005; Vazin and Freed, 2010).

The cells of the embryonic germ layers differentiate further, becoming increasingly restricted in their developmental potential. Various organs and tissues contain stem cells that are multipotent and can yield a subset of cell lineages. Further, unipotent progenitor cells are committed to giving rise to a single mature cell type. (Wagers and Weissman, 2004; Fortier, 2005; Pappa and Anagnou, 2009)

Stem cells can also be broadly classified as embryonic, fetal or adult, depending on their origin. Fetal stem cells represent an intermediate stage between embryonic and adult stem cells with high proliferation rates and differentiation potential from pluripotent -to -multipotent. Fetal stem cells can be derived either from the fetus proper or from the supportive extra-embryonic tissues of the placenta, the amniotic membrane, the amniotic fluid, umbilical cord blood and the outer region of the umbilical cord called Wharton's Jelly. (Pappa and Anagnou, 2009)

Adult stem cells can be harvested from any postnatal organism. They maintain tissue and organ mass during normal cellular turnover or in response to tissue damage having a resident population of multipotent stem cells contributing only to the parent tissue line (Fortier, 2005). Adult stem cells have been isolated from most adult tissues with hematopoietic stem cells of the bone marrow being the best-characterized adult stem cells in humans. Adult stem cells enable the ethically acceptable isolation of autologous cells and often show considerable plasticity, making these cells an attractive alternative for applications of regenerative medicine. (Korbling and Estrov, 2003; Mimeault and Batra, 2006)

## 2.2 Human pluripotent stem cells

### 2.2.1 Derivation of human embryonic stem cells

The hESC lines are derived from surplus human embryos produced by *in vitro fertilization* (IVF) for clinical purposes and donated for research by voluntary couples (Skottman, 2010). The hESCs are derived from early blastomeres (Klimanskaya et al., 2006) or morula stage (Strelchenko et al., 2004), but most often blastocyst stage embryos (Figure 1A). The ICM of the blastocyst is traditionally isolated by a method called immunosurgery using pronase, mouse anti-human antibodies and guinea pig complement, or, for example, by mechanical isolation (Thomson et al., 1998; Strom et al., 2007; Skottman, 2010). The isolated ICM is then placed on and repeatedly passaged to a layer of mitotically inactivated fibroblast feeder cells to establish a stable cell line.

The first ES cells were derived from mouse embryos in the early 1980's (Evans and Kaufman, 1981) followed by the isolation of the first human ICM cells in 1994

(Bongso et al., 1994). The first five stable hESC lines however, were not established until 1998 by James Thomson and co-workers (Thomson et al., 1998), followed by the derivation of two additional lines by Reubinoff and co-workers two years later (Reubinoff et al., 2000). Since then, hundreds of hESC lines have been established around the world. Currently 1210 hESC lines (October, 2012) have been registered at the International Stem Cell Registry database of the University of Massachusetts Medical School (http://www.iscr-admin.com/). Similarly the European Human Embryonic Stem Cell Registry - hESCreg, holds a registry of over 650 hESC lines (http://www.hescreg.eu/).

### 2.2.2 Establishing human induced pluripotent stem cells

Despite the decrease in developmental potential during differentiation, adult somatic cells can be reset back to a pluripotent state. This can be achieved through cell fusion with a pluripotent cell or by somatic cell nuclear transfer (SCNT) to an enucleated oocyte (Figure 1B), as demonstrated with the famous cloning of Dolly the sheep in 1997 by Wilmut and colleagues (Wilmut et al., 1997). However, cells generated by fusion contain a tetraploid chromosome whereas human cloning is technically very challenging and ethically debatable and thus prohibited in most countries (Yamanaka, 2008; Yamanaka and Blau, 2010). In 2006, Takahashi and Yamanaka were able to directly reprogram mouse fibroblasts back to pluripotent state by retroviral transduction and over-expression of four transcription factors: c-Myc, Oct-3/4, Sox-2, and Klf-4 (Yamanaka and Takahashi, 2006). Human iPSCs were generated the following year from human dermal fibroblasts by several research groups including Yamanaka's, using viral transduction of either the same four transcription factors (Takahashi et al., 2007; Park et al., 2008) or a set of Oct-3/4, Sox-2, Nanog, and Lin-28 (Yu et al., 2007) (Figure 1C). The mechanism of pluripotency induction is not fully understood. The core pluripotency transcription factors synergistically up-regulate ‘‘stemness’’ genes and suppress differentiation-associated genes. The early reprogramming process is accompanied by DNA hypomethylation of pluripotency-related gene promoters (Takahashi et al., 2007; Mattout et al., 2011) as well as initiation of mesenchymal-to-epithelial transition

(MET) (Mah et al., 2011) and changes in mitochondrial metabolism (Kelly et al., 2011).

The iPSC technology enabled the generation of donor autologous cells with similar phenotype and developmental potential to ESCs but without the requirement for an embryo. Human iPSCs have now been generated from different cell types e.g. adipose stem cells (Sun et al., 2009), and amniotic epithelial cells (Easley et al., 2012) with greatest reprogramming efficiency from stem or progenitor cells types (Yamanaka and Blau, 2010). Disease specific hiPSC lines have also been generated to model diseases like Huntington's disease (An et al., 2012) and long QT syndrome (Moretti et al., 2010).

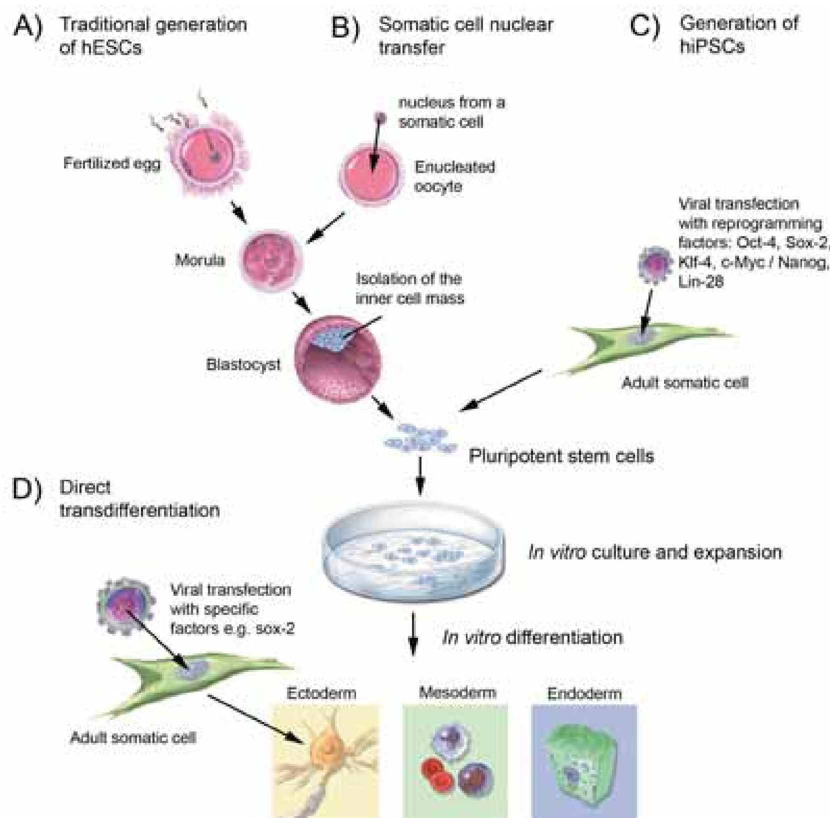


Figure 1. Some of the methods to create pluripotent cells include A) isolation of the inner mass cells from surplus embryos, B) cloning and C) genetic reprogramming of adult cells with a set of transcription factors. After culture and expansion the pluripotent cells can be induced to differentiate to derivative of the three germ layers. D) Recently direct transdifferentiation has enabled the reprogramming of somatic cells to other fully differentiated cell types without the intermediate pluripotent state. The figure is modified from pictures by Terese Winslow at Stem Cell Information, The National Institutes of Health resource for stem cell research (http://stemcells.nih.gov/info/Regenerative_Medicine/).

### 2.2.3 Characterization of human pluripotent stem cells

The self-renewing and pluripotent hESCs are traditionally defined by three criteria: 1) derivation from the preimplantation embryo, 2) prolonged undifferentiated proliferation *in vitro* and 3) stable developmental potential after prolonged culture to form derivatives of all three embryonic germ layers (Thomson et al., 1998). Human ESCs are characterized by their cell and colony morphology, expression of a set of markers associated with undifferentiated state as well as euploid karyotype and capacity to differentiate after prolonged culture (Hoffman and Carpenter, 2005).

Human ESCs grow in tightly compacted colonies maintaining defined borders. High nucleus to cytoplasm ratio and prominent nucleoli are typical features of individual cells (Thomson et al., 1998; Reubinoff et al., 2000). Undifferentiated hESCs show alkaline phospatase activity and express a number of cell surface markers including stage-specific embryonic antigens SSEA-3 and SSEA-4, tumor-related antigens TRA-1-60 and TRA-1-81, as well as certain cluster of differentiation (CD) molecules. Expression of SSEA-1 is up-regulated upon differentiation (Xu et al., 2001; Draper et al., 2004a; Carpenter et al., 2004; Hoffman and Carpenter, 2005). Human ESC transcription factors of octamer-binding transcription factor Oct-3/4 also known as POU5F1, homeodomain protein Nanog, and sex determining region Y (SRY)-box-2 gene Sox-2 form the core regulatory circuit to maintain pluripotency (Boyer et al., 2005). Other stemness markers of hESCs include: undifferentiated embryonic cell transcription factor 1 (UTF-1), zinc finger protein 42 (ZFP-42 also called REX-1); Lin-28 homolog A (Lin-28); signal transducer and activator of transcription 3 (STAT-3); left-right determination factor 1 (Lefty-1), DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B), and teratocarcinoma-derived growth factor 1 (TDGF1, also called cripto) (Sato et al., 2003; Bhattacharya et al., 2004; Richards et al., 2004; Hoffman and Carpenter, 2005). Moreover, hESCs show high telomerase activity and normal diploid karyotypes (46 XX/XY) in G-banding (Thomson et al., 1998; Reubinoff et al., 2000). Although considered karyotypically stable, hESCs undergo genomic alterations in response to prolonged culture (Draper et al., 2004b; International Stem Cell Initiative et al., 2011). Human ESCs typically also show unique epigenetic signature of open chromatin structure and specific DNA methylation and histone modification patterns (Bibikova et al., 2006).

Human ESC pluripotency is generally examined by the potential of the cells to differentiate into all three germ layers (ectoderm, mesoderm and endoderm) *in vitro* as well as *in vivo*. *In vitro*, hESCs are allowed to differentiate randomly as aggregates of cells in suspension culture called embryoid bodies (EBs). The EBs are further analyzed for gene or protein expression associated with the three germ layers. The *in vivo* test for pluripotency of hESCs is teratoma formation in severe combined immunodeficient (SCID) mice (Itskovitz-Eldor et al., 2000).

The hiPSCs have identical phenotype with the hESC counterparts. They show similar morphology and express ES cell genes and cell surface markers. The transduced pluripotency genes are initially highly active but silenced after a few passages as the endogenous pluripotency genes are reactivated in the cells. Human iPSCs show high proliferation capacity and telomerase activity, normal karyotypes and differentiation capacity similar to that of hESCs (Takahashi et al., 2007; Yu et al., 2007). Human iPSCs were originally described as indistinguishable from hESCs with similar global gene-expression patterns and epigenetic status, but recent studies have reported the two pluripotent cell types to have unique gene expression signatures with late passage hiPSCs more closely related to hESCs than early passage hiPSCs (Chin et al., 2009). Moreover, hiPSCs show residual gene expression patterns related to the tissues they are derived from (Marchetto et al., 2009; Ohi et al., 2011).

### 2.2.4 Directed *in vitro* differentiation

Theoretically, hPSCs possess the potential to produce all somatic cell types present in the adult body. However, the complex mechanisms of stem cell fate decisions, differentiation, tissue generation and organogenesis are far from fully understood. Lineage restriction and the induction of differentiation to produce specific cell types requires a complex interplay between graded concentrations of several patterning cues under temporal constraints. As the generation of EB structures has a general inductive influence, it is frequently used as a first step for *in vitro* differentiation of many cell lineages. A small fraction of cells with a particular, desired phenotype is then selected and enriched. Sequential addition of inducing growth factors and other small molecules to mimic *in vivo* embryonic developmental programs, as well as co-

culture with other cell types are common differentiation strategies. (Vazin and Freed, 2010)

Human ESCs have been differentiated to germ cells (Clark et al., 2004), likewise endodermal derivatives like hepatocytes (Schwartz et al., 2005), insulin producing β cells (Brolen et al., 2005), and lung epithelium (Samadikuchaksaraei et al., 2006). Mesodermal derivatives of chondrocytes, osteocytes, skeletal myoblasts (Barberi et al., 2005), contracting cardiomyocytes (Kehat et al., 2001), hematopoietic cells (Kaufman et al., 2001), and endothelial cells (Levenberg et al., 2002) have been generated. Various ectodermal derivatives including epidermal keratinocytes (Metallo et al., 2008), different types of neurons (Reubinoff et al., 2001; Li XJ et al., 2005), retinal cells like photoreceptors (Osakada et al., 2008) and RPE cells (Klimanskaya et al., 2004) have been generated.

While hESCs are considered to be the gold standard of pluripotency, the hiPSCs enable the generation of patient-specific and disease-specific cells from any adult individual with perfect histocompatibility match (Puri and Nagy, 2012). Large-scale differentiation of hPSCs to different functional cell types provides a valuable tool to study embryonic development and differentiation. In addition these cells can offer model systems for novel pharmaceutical drug discovery assays, as well as new drug metabolism and cytotoxicity screens. Most importantly they can serve as a potentially inexhaustible source of cells for regenerative cell replacement therapies. Many adult cell types, such as neural cells and cardiomyocytes, have low recovery and proliferative capacity, combined with a shortage of donated tissue available. Human PSC-derived cells can thus provide novel treatment options for devastating conditions like spinal cord injuries or myocardial infarction. (Vazin and Freed, 2010)

Recently adult somatic cells have been directly reprogrammed without a pluripotent intermediate, i.e. transdifferentiated into other fully differentiated cell types by over-expression of key transcription factors (Cohen and Melton, 2011) (Figure 1D). For example, cardiomyocytes (Ieda et al., 2010) have been generated by direct transdifferentiation and human fibroblasts have been induced to neural stem cells even with a single factor of Sox-2 (Ring et al., 2012).

## 2.3 *In vitro* culture of undifferentiated human pluripotent stem cells

Initially the hESCs were derived and cultured *in vitro* on mouse embryonic fibroblast (mEF) feeder cells in a culture medium supplemented with fetal bovine serum (FBS) (Thomson et al., 1998; Reubinoff et al., 2000). Constant and optimal culture conditions are required for the maintenance of the pluripotent, undifferentiated hESC status and prevention of spontaneous differentiation. The hESCs need continuous, weekly passaging to freshly prepared feeder cell layers as well as daily medium changes. The passaging is done either manually by cutting colony pieces or by using different enzymatic techniques. The hiPSCs are cultured in a similar manner to hESCs. The *in vitro* culture of the hPSCs is thus labour intensive, expensive and contains undefined, animal derived compounds. The use of xenogeneic material poses safety risks for downstream clinical applications of stem cells as discussed later in Chapter 2.4.3. Rigorous research in recent years has focused on finding simpler, defined and xeno-free hESC culture conditions.

### 2.3.1 Human pluripotent stem cell culture media for feeder-dependent culture

The culture conditions used for the initial derivation of hESCs included culture medium supplemented with high levels (20%) of fetal bovine serum (FBS) (Thomson et al., 1998). FBS is not only xenogeneic but also variable in its components and quality, resulting in significant lot-to-lot variability. The hESC culture is also negatively affected by FBS supplementation that leads to a significant reduction in growth and excessive differentiation (Amit et al., 2000; Koivisto et al., 2004). Human serum (HS) has been used to replace FBS in hESC culture and has been shown to enable both derivation and long-term culture of hESCs (Richards et al., 2002; Ellerstrom et al., 2006). Although HS provides a xeno-free serum alternative, as FBS it is undefined in composition and subjected to batch variability.

An important improvement in the culture conditions was the replacement of sera with 20% Knockout™ Serum Replacement (ko-SR, Life Technologies) and basic fibroblast growth factor (bFGF) (Amit et al., 2000). The serum-free culture medium

enabled long-term culture of hESCs with substantially less spontaneous differentiation and higher growth rate, as well as several-fold increase in cloning efficiency compared to culture with FBS. The addition of 4-8 ng/ml bFGF to the ko-SR medium was required for continued undifferentiated proliferation of hESCs (Amit et al., 2000; Koivisto et al., 2004). The ko-SR culture medium also supports the derivation of new hESC lines and has become a widely used standard for hESC cultures (Genbacev et al., 2005; Inzunza et al., 2005; Lysdahl et al., 2006). Ko-SR contains xeno-compounds like Albumax, a lipid rich bovine serum albumin (BSA) which, in addition to lipids, also carries undefined impurities, cytokines, hormones, and growth inhibitors (Price et al., 1998). Most of the hESC culture media used for either feeder-dependent or independent hESC culture contain bovine or human serum albumin (HSA) products as carrier components.

Serum-free and xeno-free serum replacers and media alternatives for hESC culture have been developed during recent years as mechanisms of hESC pluripotency and self-renewal have been elucidated. Ko-SR is currently available as a xeno-free alternative. A plant derived serum replacement VegetaCell has been used for long-term hESC maintenance in combination with HSA and human feeder cells as well as for feeder-independent culture (Kunova et al., 2010). A serum-free, fully defined medium containing vitronectin, insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3) and bFGF has been shown to enable hESC propagation for ten passages but the culture required use of mEF feeder cells (Richards et al., 2008). Other examples of xeno-free media intended for feeder-dependent culture are HEScGRO™ medium (Millipore) and RegES medium (Rajala et al., 2010).

The first hiPSC lines were cultured similarly to hESC counterparts in ko-SR containing media or Primate ES medium (ReproCELL, Japan) supplemented with bFGF (Takahashi et al., 2007). In general the media used for hESC culture also enables hiPSC culture. The hPSC culture media development has primarily focused on feeder-independent culture systems.

### 2.3.2 Feeder cell culture

Originally hESCs were established on mitotically inactivated mEF feeder cells that were cultured in FBS-containing culture medium (Thomson et al., 1998). Mouse EFs are derived from the small early murine fetuses and are a mixture of fetal cells. They are thus subject to large batch-to-batch variability with variable capacity to support hESC lines. Moreover, the lifespan of mEFs is relatively short as they undergo senescence after five to six passages after derivation (Richards et al., 2002; Amit et al., 2003). A substantial work load is associated with the isolation and culture of mEF feeder cells. To overcome this problem, immortalized mEF line called STO feeder cells have been used instead of primary mEFs (Park et al., 2004).

#### *2.3.2.1 Human feeder cells*

Various human feeder cells have been widely tested for hESC propagation. Richards and co-workers were the first to report long-term undifferentiated culture of two hESC lines on feeder layers derived from human fetal muscle, fetal skin, and adult fallopian tube epithelial cells. The fibroblasts were derived and cultured using HS-containing culture medium (Richards et al., 2002). A year later the same research group comparatively evaluated and ranked a panel of 11 different human feeder cells. Human fetal muscle and skin derived fibroblasts were reported as most supportive of hESCs. Most adult derived fibroblasts were unsupportive of hESCs and only those derived from skin biopsies maintained long-term undifferentiated culture, still being inferior to the feeder cells of fetal origin (Richards et al., 2003). Human embryonic fibroblasts have since been used for hESC derivation and culture with great success (Chavez et al., 2008; Zhou et al., 2008; Kumar et al., 2009; Kibschull et al., 2011). The ethical issues related to using aborted human fetuses led to search for other tissue sources. Hovatta and co-workers reported derivation of a new hESC line on postnatal human foreskin fibroblast (hFF) feeder cells (Hovatta et al., 2003). Human FFs are simple to derive and can be continuously cultured for up to 42 passages without reduction in the rate of growth or ability to support hESCs (Amit et al., 2003). Both in-house-derived and commercial hFFs have since been widely used (Choo et al., 2004; Inzunza et al., 2005; Ellerstrom et al., 2006; Unger et al., 2009; Aguilar-Gallardo et al., 2010; Skottman, 2010).

Other human sourced feeder cells include, for example, bone marrow stromal cells (Cheng et al., 2003), umbilical cord-derived cells (Zhan et al., 2008; Cho et al., 2010; Tannenbaum et al., 2012), uterine endometrial cells (Lee et al., 2005), placental cells (Genbacev et al., 2005; McKay et al., 2011), and adipose mesenchymal stem cells (Cortes et al., 2009). Genetically modified fibroblasts have been used for more efficient and scalable propagation (Unger et al., 2009; McKay et al., 2011). Fibroblast-like cells derived from the spontaneous differentiation of hESCs (hESC-df) offer a genotypically homogenous and stable feeder cell population, however, the initial establishment of hESC lines remains unsolved for these culture systems (Stojkovic et al., 2005a; Wang Q et al., 2005; Chen HF et al., 2009; Lee et al., 2012). Human iPSCs generated from adult dermal tissue can be maintained on autologous feeder layers with the non-reprogrammed cells serving as feeders (Takahashi et al., 2009) or isogenic, inactivated feeder layers can be prepared from the cells used as source for hiPSC derivation (Unger et al., 2009; Rodriguez-Piza et al., 2010; Zou et al., 2012). Some of the human feeder cell types as well as media used for fibroblast and hPSC cultures are summarized in Table 1.

Table 1. Human feeder-dependent hPSC culture systems.

| Feeder cell source | Key feeder medium components | hPSC | Key hPSC medium components | Publication |
|---|---|---|---|---|
| Fetal muscle (I)<br>Fetal skin (I)<br>Adult fallopian tube (I) | FBS/HS | hESC (M)<br>hESC (M)<br>hESC (D) | FBS<br>FBS<br>HS | Richards et al. 2002 |
| Fetal skin (C)<br>Adult skin (I) | FBS/HS | hESC (M)<br>hESC (M) | FBS, ITS/<br>ko-SR, bFGF | Richards et al. 2003 |
| Foreskin (I) | FBS/HS/<br>ko-SR | hESC (M) | ko-SR, bFGF | Amit et al. 2003 |
| Bone marrow stroma (I) | FBS, bFGF | hESC (M) | ko-SR, bFGF | Cheng et al. 2003 |
| Foreskin (C) | FBS | hESC (D) | FBS, LIF | Hovatta et al. 2003 |
| Foreskin (C) | FBS | hESC (M) | FBS/ ko-SR,<br>bFGF | Choo et al. 2004 |
| Placenta (I) | FBS | hESC (D) | ko-SR, bFGF | Genbacev et al. 2005 |
| Uterine endometrium (I) | FBS | hESC (D) | ko-SR, bFGF | Lee et al. 2005 |
| hESC-dF (I) | FBS | hESC (M) | ko-SR, bFGF | Stojkovic et al. 2005a |
| hESC-dF (I) | FBF/ HS,<br>ITS, EGF | hESC (D) | ko-SR, bFGF | Wang Q et al. 2005 |
| Foreskin (I) | HS | hESC (D) | HS, bFGF | Ellerström et al. 2006 |
| Foreskin (I) | HS/<br>LiforCell® | hESC (M) | HEScGRO™ | Meng et al. 2008 |
| hESC-dF (I) | HS/ko-SR/<br>N2,ITS,<br>bFGF | hESC (M) | ko-SR, bFGF/<br>1% HS, bFGF,<br>noggin, activin A | Chen HF et al. 2009 |
| Foreskin (C) | FBS | hESC (M) | HEScGRO™/<br>ko-SR<br>ko-SR (xeno-free) | Chin et al. 2010 |
| Foreskin (I)<br>Adult skin (I) | HS | hESC (M)<br>hiPSC source (D)<br>and autologous<br>feeders (M) | F44, bFGF/<br>ko-SR (xeno-<br>free), ko-SR<br>Growth Factor<br>Cocktail (xeno-<br>free), bFGF | Rodriguez-Piza et al.<br>2010 |
| Adult skin (C) | FBS | hiPSC source (D)<br>and autologous<br>feeders (M) | ko-SR, bFGF | Takahashi et al. 2009 |
| Foreskin (C)<br>(genetically modified) | FBS | hESC (M)<br>hiPSC source (D)<br>and autologous<br>feeders (M) | ko-SR, bFGF | Unger et al. 2009 |
| Umbilical cord stroma (I) | FBS | hESC (M) | ko-SR, bFGF | Cho et al. 2010 |
| Embryonic connective<br>tissue (I) | HS, bFGF | hESC (M) | HEScGRO™,<br>ROCKi | Kibschull et al. 2011 |
| Foreskin (C) | FBS | hESC (D) | RegES<br>(xeno-free) | Rajala et al. 2010 |
| Adult skin (C) | FBS | hESC (D) | ko-SR, bFGF | Tecirlioglu et al. 2010 |
| Placenta (I)<br>(genetically modified) | HS | hESC (D)<br>hiPSC (M) | ko-SR, bFGF | McKay et al. 2011 |
| Bone marrow mesenchymal<br>stem cells<br>(genetically modified) (I) | FBS | hESC (M)<br>hiPSC source (D)<br>and autologous<br>feeders (M) | ko-SR, bFGF | Zou et al. 2012 |

Abbreviations: I, isolation of feeder cells in-house; C, commercial feeder cells used; D, derivation of a new hPSC line; M, maintenance of a previously derived hPSC line; N2, chemically defined neuronal cell supplement (Lifetechnologies); LiforCell®, serum and animal protein free serum replacement (Lifeblood Medical); HEScGRO™, xeno-free hESC culture medium (Millipore); RegES, xeno-free hESC culture medium; F44, human plasma-derived cell culture additive. Other abbreviations are presented on page 13.

#### 2.3.2.2 *Feeder cell culture media*

Even the human feeder cell culture is typically based on the use of FBS containing feeder cell culture medium. For xeno-free fibroblast culture, HS has been used as a substitute (Richards et al., 2003; Meng et al., 2008; Unger et al., 2008a; Kibschull et al., 2011; Rodriguez-Piza et al., 2010; Ross et al., 2010) and was used to derive xeno-free feeders for the first xeno-free hESC line by Ellerström and co-workers (Ellerstrom et al., 2006). The use of ko-SR has been reported for the derivation of fibroblast by Amit and co-workers but these results have not been verified by other groups and the manufacturer states that ko-SR is not suitable for fibroblast propagation (Amit et al., 2003). Ko-SR as well as a combination of N2, insulin/transferrin/selenium (ITS) supplements and bFGF has been used to establish and propagate autologous feeder cells from hESCs (Chen HF et al., 2009). Low serum and serum-free and even xeno-free media formulations for different cell types, including fibroblasts, are currently available from many cell culture media suppliers. Examples are: FibroGRO™-LS Complete Media Kit (Millipore, MA, USA); FibroLife® Serum-Free Cell Culture Medium for fibroblasts (Lifeline Cell Technology, MD, USA); Medium 106 for the culture of dermal fibroblasts combined to Low Serum Growth Supplement containing 2% FBS and growth factors (Life Technologies, Paisley, UK); Fibroblast Medium-animal component free (FM-acf) (ScienCell Research Laboratories, CA, USA) and CnT-DP-3D, a fully defined medium for fibroblast culture (CELLnTEC Advanced Cell Systems, Switzerland). The use of serum-free media has not been reported in the context of feeder cell derivation and maintenance although some recent publications have used serum-free media to establish fibroblasts for iPSC generation (Chen et al., 2011; Macarthur et al., 2012).

#### 2.3.2.3 *Role of feeder cells*

Different types of feeder cells and even feeder cell lines originating from the same tissue source show different capacities to support undifferentiated hESC culture (Richards et al., 2003; Eiselleova et al., 2008). Similarly, dermal fibroblasts from adult donors show varying capacity as supportive feeder cells for iPSCs derived from them (Takahashi et al., 2009). It has been reported that the use of feeder cells

does not accelerate reprogramming or increase the frequency of mouse iPSC colonies but does improve the growth of the primary iPSC colonies after reprogramming (Chen M et al., 2009). It is clear that the feeder cells have a crucial role in maintaining undifferentiated hPSC morphology and pluripotent status in feeder-dependent culture conditions, but their role and contribution is not fully understood. The feeder cells provide the hPSCs with an attachment and growth substrate and secrete various growth factors, cytokines and adhesion-related proteins that promote pluripotency. The feeder contribution has been studied by transcriptome analysis of hESC supportive and non-supportive feeder cells and immuno- and proteome analyses of the secreted factors in medium conditioned (i.e. incubated overnight) on feeder cells. These studies have revealed that the human feeder cells secrete low levels of bFGF, insulin growth factor binding proteins (IGFBP) and proteins involved in transforming growth factor beta (TGF-ß) superfamily signaling like TGF-ß, low levels of activin A, TGF-ß-binding proteins and antagonistic follistatin, as well as bone morphogenetic protein (BMP) antagonist Gremlin. The mEF cells secrete pigment epithelium derived factor (PEDF), IGF and IGF family proteins as well as higher levels of Activin A compared to human feeder cells. In addition the feeder cells secrete various extracellular matrix (ECM) components like collagens, laminins, fibronectin, heparan sulfate proteoglycans (HSPG) like perlecan and ECM remodeling proteins (Lim and Bodnar, 2002; Prowse et al., 2005; Kueh et al., 2006; Chin et al., 2007; Prowse et al., 2007; Bendall et al., 2007; Eiselleova et al., 2008; Levenstein et al., 2008; Bendall et al., 2009).

### 2.3.3 Feeder-independent culture conditions

#### *2.3.3.1 Conditioned media and undefined matrices*

The culture medium and the substrata together provide the hPSCs with nutrient components, growth factors, ECM and other molecules to enable proper attachment and correct signaling for the maintenance of pluripotency. The first feeder-independent culture system for hESCs utilized mEF conditioned medium (mEF-CM) combined with BD Matrigel™ Basement Membrane Matrix (Matrigel™, BD

Biosciences, NJ, USA) (Xu et al., 2001). The fibroblast CM is prepared by incubating standard hESC culture medium (ko-SR, bFGF) on a confluent layer of inactivated fibroblasts to enable secretion of growth and pluripotency promoting factors to the culture medium, which is thereafter used for hESC culture. The use of CM eliminates direct contact to feeder cells but still requires large scale propagation of feeder cells and introduces an undefined xeno-component to the culture system. Similarly Matrigel™ is a mouse sarcoma derived basement membrane preparation containing a mixture of ECM proteins such as laminins, collagen IV, entactin, HSPGs, and growth factors (Kleinman et al., 1982).

The combination of Matrigel™ and mEF-CM has been widely used for feeder-independent hESC culture (Brimble et al., 2004; Carpenter et al., 2004; Rosler et al., 2004) and for feeder-independent hiPSC culture as described in conjunction with the first derivations (Takahashi et al., 2007). Other types of CM e.g. hESC derived autologous feeder cell-CM (hESCdf-CM) were described in early studies (Xu et al., 2004; Stojkovic et al., 2005a). CM has even enabled a completely matrix-free hESC culture directly on tissue culture polystyrene (Bigdeli et al., 2008; Mahlstedt et al., 2010). In addition to Matrigel™, human and bovine sera coatings were introduced as culture substrata since serum is rich in ECM proteins (Stojkovic et al., 2005b; Vallier et al., 2005).

#### *2.3.3.2 Development of more defined culture systems and maintenance of pluripotency*

As the feeder-independent culture conditions were described for hESCs, it became possible to systematically define and characterize the exogenous factors required for maintenance of undifferentiated, pluripotent hESC growth. Numerous studies have been conducted to test various combinations of growth factors and other media supplements to create defined and eventually xeno-free culture media. Simultaneously the culture substrata used have shifted from animal ECM preparations and sera coatings to human sourced or recombinant ECMs and eventually to synthetic and engineered substrata. Some of these feeder-independent culture systems are described in Table 2.

Table 2. Human feeder-independent hPSC culture systems.

| Substrate | hPSCs | Key hPSC medium components | Publication |
|---|---|---|---|
| Matrigel™/laminin | hESC (M) | mEF-CM, bFGF | Xu et al. 2001 |
| Matrigel™ | hESC (M) | hESC-df-CM (genetically modified) | Xu et al. 2004 |
| Human and bovine fibronectin | hESC (M) | 15% ko-SR, TGF-ß, low bFGF, (LIF) | Amit et al. 2004/Amit and Itskovitz-Eldor, 2006 |
| Laminin | hESC (M) | ko-SR, high activin A, NIC, KGF | Beattie et al. 2005 |
| mEF ECM | hESC (D) | 8% ko-SR, 8% Plasmanate®, LIF, bFGF | Klimanskaya et al. 2005 |
| Human laminin | hESC (M) | X-Vivo™ 10, high bFGF, (flt3 ligand) | Li Y et al. 2005 |
| HS | hESC (M) | hESC-df – CM, bFGF, ITS | Stojkovic et al. 2005b |
| FBS | hESC (M) | CDM: albumin, insulin, transferrin, monothioglycerol, bFGF, activin A | Vallier et al. 2005 |
| Matrigel™ | hESC (M) | ko-SR, high FGF, (Noggin) | Xu C et al., 2005 |
| Human laminin | hESC (D) | X-Vivo™ 10, high bFGF | Fletcher et al. 2006 |
| Matrigel™/human fibronectin | hESC (M) | N2, B27, high bFGF | Liu et al., 2006 |
| Fibronectin | hESC (M) | HESCO: albumin,cholesterol, insulin, transferrin, bFGF, Wnt3a, April/BAFF, | Lu et al. 2006 |
| hECM mix | hESC (D) | TeSR1 (xeno-free) | Ludwig et al. 2006a |
| Matrigel™ | hESC (M) | mTeSR™1 | Ludwig et al. 2006b |
| Matrigel™ | hESC (M) | BSA, N2, B27, bFGF | Yao et al. 2006 |
| Matrigel™ | hESC (M) | ko-SR, low activin A | Xiao et al. 2006 |
| Matrigel™/HS/human fibronectin | hESC (M) | DC-HAIF (StemPro® hESC SFM): BSA, ascorbic acid, transferrin, trace elements, IGF1, heregulin-1ß, bFGF, activin A | Wang et al. 2007 |
| Matrigel™ | hiPSC (M) | mEF-CM | Takahashi et al., 2007 |
| Human vitronectin (rh- or natural) | hESC (M) | mTeSR™1 | Braam et al. 2008 |
| hMSC ECM | hESC (M) | SBX medium, bFGF, high ActA/ low TGF-ß | Peiffer et al. 2008 |
| Matrigel™ | hiPSC (D, hASC) | FBS (for D)/ mTeSR™1 (for M) | Sun et al. 2009 |
| E-cadherin /IgG fusion protein | hESC (M) hiPSC (M) | mTeSR™1 | Nagaoka et al. 2010 |
| rh-Laminin-511 | hESC (M) hiPSC (M) | TeSR™1 | Rodin et al. 2010 |
| CELLstart™ (xeno-free) | hiPSC (D, hASC) | StemPro® MSC SFM xeno-free/ 2% HS (for D) NutriStem™ XF/FF Culture Medium (for M) (all xeno-free) | Sugii et al. 2010 |
| Human recombinant vitronectin | hESC (M) hiPSC (D, hDF) | E8 | Chen et al. 2011 |
| PMEDSAH | hESC (M) | StemPro® hESC SFM | Nandivada et al. 2011 |

Abbreviations: D, derivation; M, maintenance; X-Vivo™ 10, defined, xeno-free medium (Cambrex Bioscience); NutriStem™ XF/FF Culture Medium, xeno-free hPSC culture medium (Stemgent); HESCO, hESC Cocktail; DC-HAIF: a defined hESC culture medium; hMSC ECM, human mesenchmal stem cell derived matrix; SBX medium, defined medium with synthetic lipid and iron carriers instead of albumin (AxCell); hASC, human adipose stem cells; rh-, recombinant human; CELLstart™, xeno-free hPSC culture matrix (Lifetechnologies); Plasmanate®, human plasma derived cell culture additive (Bayer Helthcare); TeSR1, xeno-free hESC culture medium; mTeSR™1, xeno-compound containing modified TeSR1 (STEMCELL Technologies); N2, chemically defined neuronal cell supplement (Life Technologies); B27, serum-free neuronal cell supplement (Life Technologies); StemPro® MSC SFM xeno-free, xeno-free, serum-free culture medium for mesenchymal stem cells (Lifetechnologies); StemPro® hESC SFM; serum-free culture medium for hESCs (Life Technologies); E8, xeno-free, albumin free hPSC culture medium; PMEDSAH; poly[2-(methacryloyloxy)ethyl-dimethyl-(3-sulfopropyl)ammonium hydroxide]. Other abbreviations are presented on page 13.

The ECM proteins are important *in vivo* niches of stem cells regulating central cellular processes such as cell proliferation, differentiation and migration, serve as a reservoir for growth factors and provide a substrate for attachment and spreading (Chen et al., 2007). Human ESCs produce different ECM proteins and, for example, laminins, collagens, fibronectin, vitronectin and E-cadherin have been used as hESC substrata indicating their central role for hESC culture. Laminins, the main components of basement membranes, are trimeric glycoproteins consisting of one α (1–5), one ß (1–3), and one γ (1–3) chain (Colognato and Yurchenco, 2000). Vitronectin is an ECM glycoprotein containing multiple domains capable of binding e.g. collagens and integrins. The N-terminal domain of vitronectin contains an Arg-Gly-Asp (RGD) sequence known to bind cell surface receptors (Furutani et al., 2012). E-cadherin is a transmembrane glycoprotein and cell adhesion molecule posed to contribute to hESC self-renewal (Li L et al., 2012). The ECM interacts with cells via cell surface receptors, mainly integrins, that are heterodimeric cell membrane receptors of one α (1–18) and one ß (1–8) subunit (Hynes, 2002). Human ESCs express high levels of integrin α subunits α3, α6, α7 (laminin binding), α5, αV (fibronectin/vitronectin (RGD, Arginine-Glycine-Aspartic acid binding) and low levels of α1, α2 subunits (collagen binding) (Xu et al., 2001; Miyazaki et al., 2008; Vuoristo et al., 2009).

In addition to the culture substrate, the medium provides the hPSCs with the essential signaling factors to maintain pluripotency. Unlike mouse counterparts, hESC self-renewal is not affected by the addition of leukemia inhibitory factor (LIF) or the resultant Janus Kinase (JAK)/signal transducers and activators of transcription 3 (STAT3) activation (Xu et al., 2002; Daheron et al., 2004; Humphrey et al., 2004). The FGF, TGF-ß and Wnt pathways have central roles in the self-renewal of hESCs and have been extensively studied. Basic FGF has been an important culture additive from early on in both feeder-dependent and independent culture systems. Undifferentiated hESCs express bFGF and high-affinity FGF receptors: FGFR1, FGFR3, and FGFR4. Stimulation of hESCs by bFGF leads to tyrosine phosphorylation of various proteins and to activation of extracellular signal regulated kinases, in particular ERK1/2. (Sato et al., 2003; Sperger et al., 2003; Dvorak et al., 2005). The addition of high levels of bFGF (40-100 ng/ml) have been shown to support undifferentiated hESC culture on Matrigel™ alone (Xu C et al.,

2005) or in combination with BMP antagonist noggin (Wang G et al., 2005; Xu RH et al., 2005).

The TGF-ß superfamily members, including the TGF-ß/activin/nodal branch and the BMP/growth and differentiation factor (GDF) family are also important regulators of hESC pluripotency. The effects of TGF-ß signaling are mediated by binding to cell surface type I and type II receptors leading to the phosphorylation of intracellular receptor-Smads. Upon phosphorylation, the Smads associate with Smad4, localize to the nucleus and activate transcription. Activin, nodal and TGF-ß signal through Smad2 and Smad3, whereas BMP/GDF signals through Smads 1, 5, and 8. Maintenance of hESCs is associated with active Smad2/3 signaling and the suppression of BMP signaling mediated through Smads 1/5/8. (James et al., 2005; Peerani et al., 2007). Accordingly, Amit and co-workers showed that TGF-ß1 combined with bFGF could support prolonged undifferentiated culture of hESCs in ko-SR containing medium on fibronectin matrices (Amit et al., 2004; Amit and Itskovitz-Eldor, 2006). Vallier and co-workers reported that activin/nodal and FGF in combination maintain hESC pluripotency in the absence of feeder cells. They described a culture system on FBS coated surfaces combined with chemically defined medium (CDM) (Vallier et al., 2005).

The main components of the canonical Wnt pathway are detected in undifferentiated hESCs (Sato et al., 2003), and the Wnt pathway activation by 6-bromoindirubin-3′-oxime (BIO) or culture supplementation by Wnt3 has been reported to be sufficient to maintain undifferentiated hESC phenotype (Sato et al., 2004), but contradictory reports have concluded that Wnt activation is incapable of maintaining undifferentiated hESC expansion (Dravid et al., 2005).

Another key player for hESC self-renewal is the insulin growth factor (IGF) pathway. Ko-SR contains insulin (Price et al., 1998) and insulin is included in most hESC culture media formulations. IGF2 is secreted by mEFs and autologous fibroblast-like cells originating at the hESC colony edges (autologous feeder cells) as a response to exogenous bFGF, and IGF2 alone has been shown to be sufficient to maintain hESC cultures (Bendall et al., 2007). Wang and co-workers demonstrated that hESC self-renewal requires insulin-like growth factor and epidermal growth factor (ERBB2) receptor signaling (Wang et al., 2007). They developed a defined medium called DC-HAIF that supported the long-term culture of several hESC lines. The DC-HAIF medium was later commercialized and is

available as a formulation called StemPro® hESC SFM. The medium is serum, but not xeno-free and marketed in combination with xeno-free CELLstart™ matrix (both from Life Technologies).

Ludwig and colleagues were able to derive two new hESC lines on a mixture of human ECM proteins of laminin, collagens, fibronectin and vitronectin in combination with a novel serum and xeno-free culture medium called TeSR1 (Ludwig et al., 2006a). The development of the medium included extensive testing of the physicochemical environment, growth factor supplements and matrix components. The final medium included DMEM/F12 base supplemented with HSA, vitamins, antioxidants, trace minerals, specific lipids and cloned growth factors such as high concentration of bFGF. Unfortunately the two new hESC lines derived were found to be karyotypically abnormal after a few months of culture in the culture system. A xeno-compound containing, more affordable version of the media for research grade hESC culture was published the following year (Ludwig et al., 2006b). This modified TeSR1 (mTeSR™1) was used in combination with Matrigel™ and is commercially available from STEMCELL Technologies. The culture system have since been shown to enable feeder-independent derivation of new hESC lines with the aid of anti-apoptotic Rho-associated kinas inhibitor ROCKi (Lagarkova et al., 2010) and both hESC and hiPSC culture combined to defined substrates (Braam et al., 2008; Nagaoka et al., 2010; Rodin et al., 2010). Recently a further developed simplified, albumin free version of the TeSR1 called E8 was reported to support established hESC and iPSC lines and enable efficient hiPSC derivation from dermal biopsy samples on human recombinant vitronectin surfaces (Chen et al., 2011). Other reports on feeder-independent hiPSC derivations have recently been published (Hayashi et al., 2010; Beltrao-Braga et al., 2011; Chung et al., 2012) even in xeno-free culture conditions (Sugii et al., 2010; Wang et al., 2012). Lower efficiencies compared to use of mEF feeder cells in the process have been reported, even with easily reprogrammed adipose-derived stem cells serving as the cell source (Sun et al., 2009). Also, despite the feeder-independent transduction process, later propagation often utilizes feeder cell support (Macarthur et al., 2012).

Elimination of the feeder cells from the hESC culture system substantially reduces the labor of hESC culture, offers more defined and reproducible culture systems, and enables the scale-up of hESC production for potential clinical use. The

use of xeno-free human derived or synthetic materials in culture media and as attachment substrata can then be validated to correspond to GMP-quality requirements.

## 2.4 Human pluripotent stem cells for regenerative medicine

### 2.4.1 Human pluripotent stem cell therapies

Regenerative medicine is a multidisciplinary area aiming at the maintenance, improvement, or restoration of cell, tissue, or organ function using methods of cell therapy, gene therapy, and tissue engineering (Liras, 2010). Therapies based on adult stem cells have been in use for several decades beginning with the first bone marrow hematopoietic stem cell transplants in the late 1960s (Bach et al., 1968). Some cell products for cartilage or skin replacement using autologous cells like chondrocytes or fibroblasts have appeared on the market, while hPSC applications are in the early phase of development (Carpenter et al., 2009).

The general strategy for hPSC therapies is the production of large quantities of clinical grade undifferentiated pluripotent cells, which are then differentiated into specific, functional cell type and delivered to the patient, where they integrate with the host system to replace the damaged cells and repair a tissue or restore lost or defective functions (Carpenter et al., 2009). The therapeutic effects of hESC-derived progeny have been reported in proof-of-concept preclinical studies in animal models of spinal cord injury (Keirstead et al., 2005), Parkinson's disease (Cai et al., 2009), diabetes (Kroon et al., 2008), myocardial infarction (Pal, 2009), liver disease (Basma et al., 2009) and retinal disease (Lamba et al., 2009).

In 2009, the US Food and Drug Administration (FDA) allowed the Geron Corporation (Menlo Park, CA, USA) to begin the first clinical trial using hESCs to treat patients with spinal cord injury. The strategy was based on the promotion of remyelination by transplantation of hESC derived oligodendrocyte precursor cells. A Phase 1 clinical trial was initiated but was discontinued for financial reasons in 2011 (http://cell-therapies.geron.com/). A biotechnology company Advanced Cell Technology (CA, USA) operating in both the UK and the USA recently undertook a

cell replacement trial to assess the safety of treating retinal degenerative diseases by transplantation of hESC-derived RPE cells (Schwartz et al., 2012). The use of research grade hESCs established on mEF feeder cells and using FBS, has been approved for these first clinical trials.

Since the clinical trials ongoing and under planning are phase I studies to address safety issues, it can be expected that useful clinical data on hESC-progeny therapeutic effectiveness will not be available for 5 to 10 years (Liras, 2010). The first clinical trials are being conducted with hESCs whereas hiPSC technology is still much further away from the clinical trial stage. Thus the following Chapters will mainly discuss clinical grade hESCs. The problems associated with hiPSC technology regarding their clinical use will be discussed briefly in Chapter 2.4.4.

### 2.4.2 Regulatory aspects

As technologies for cell therapies have advanced, so have the regulatory systems controlling their use. Cell therapies are regulated in Europe by the European Medicines Agency (EMEA) and in the USA by the US Food and Drug Administration (FDA). In Europe the requirements for cell therapy products are additionally outlined in several European Commission directives and guidelines (Directive 2004/23/EC, Commission Directives 2006/17/EC, 2006/86/EC, EU Regulation 1394/2007/EC2 for advanced therapy medicinal products, and EMEA guidelines on human and xenogeneic cell-based medicinal products EMEA/CHMP/410869/2006 and EMEA/CHMP/CPWP/83508/2009). As hPSC-derived products are defined as advanced therapy medicinal products, their manufacture is strictly regulated. (Unger et al., 2008b; Carpenter et al., 2009)

Human ESCs destined for clinical applications need to be derived according to Good Tissue Practice (GTP) and GMP-requirements. These strict quality assurance systems include meeting donor eligibility rules and ensuring compliance in the recovery, screening, testing, processing, storing, labeling, packaging, and distribution of cells. The goal in manufacturing a GMP-compatible hESC-derived product is the consistent generation of sufficient cell numbers in an aseptic environment with adequate safeguards, sterility, and traceability. This will require developing a process that allows adequate propagation to obtain large numbers of

undifferentiated cells, a defined method of differentiation and validated testing methods to ensure the function, composition, and batch-to-batch consistency of the final product. GMP-manufacturing requires traceability of raw materials, production under standardized operating procedures (SOPs) in clean room facilities and validated tests to monitor quality, safety, and consistency over the entire production cycle. The goal is to identify and standardize the best practices to produce safe cell products with reproducible characteristics and quality to ensure the safety of the patient. (Unger et al., 2008b; Carpenter et al., 2009; Liras, 2010).

### 2.4.3 Clinical quality culture conditions and protocols

Most of the hESC lines to date have been generated for research purposes. Xenogeneic products are present at multiple steps in current protocols for hESC derivation and maintenance including immunosurgery, use of mEF feeder cells, FBS-supplemented culture media, animal-sourced enzymes (such as trypsin, dispase, or collagenases) used for cell dissociation, and BSA in the hESC culture media. Finally, similar animal sourced materials are used for the induction of differentiation, cell maturation and delivery strategies.

Although approved from the regulatory point of few, the use of xenogeneic materials for the production of clinical grade cells is undesirable. In addition to xenogeneic pathogens like murine viruses or prions, immunogenic antigens such as apolipoprotein B-100 (apoB-100) and sialic acid N-glycolylneuraminic acid (Neu5Gc) have been shown to contaminate hESCs cultured under conventional culture conditions (Martin et al., 2005; Heiskanen et al., 2007; Hisamatsu-Sakamoto et al., 2008; Hayashi et al., 2010). Strong antibody reactions in patients after administrating of therapeutic cells cultured in FBS-containing culture medium have been reported (Mackensen et al., 2000; Sakamoto et al., 2007). Although xeno-free conditions are not a prerequisite of GMP, it is widely accepted that the clinical quality hPSC cells should also be xeno-free.

The generation of the first current GMP (cGMP)-quality hESC lines was reported in 2007 by Crook and co-workers (Crook et al., 2007). Their groundwork was an excellent starting point for establishing clinical grade hESC lines, however, the cells were not xeno-free. Recently the establishing of the first GMP-compliant and also

xeno-free hESC lines on umbilical cord fibroblasts was reported (Tannenbaum et al., 2012).

Producing the clinical quality hESC cells and the feeder cells for their support involves extensive steps of optimization, standardization, and validation. Some of the key points in establishing clinical grade hESC and feeder cells are shown in Figure 2. The process development highlighted in Figure 2 encompasses all aspects shown for feeder cell and hESC establishment.

For the feeder cells the first point to consider is the source of cells to be used. Mouse EFs are considered unfit for clinical use. The hESC cells are cultured in direct contact with the feeder cells for multiple passages which provides an ideal setting for the transfer of oncogenic viruses, which, moreover, are challenging to detect in cell cultures (Stacey et al., 2006). The choice of human tissue source should be based on a careful risk analysis and also non-pathologic tissue availability, non-invasive tissue collection procedure, issues relating to tissue transfer to the GMP-laboratory, ease and constant efficiency of feeder cell derivation and, importantly, the capacity to support hESC derivation and culture are aspects to consider. The laboratory process and optimal cell numbers for feeder cell isolation, culture, and cryostorage of master cell banks (MCB) and working cell banks (WCB) need to be optimized. Additionally, the process of preparing feeder cells for hESC co-culture include the manner of inactivation, cell plating density, media changes and adaption, hESC seeding, and co-culture. All materials should be of cGMP-quality including optimally GMP-quality, xeno-free and defined feeder cell culture and cryomedia. Also, the feeder cell lines established need to undergo excessive biosafety testing and characterization. (Stacey et al., 2006; Crook et al., 2007; Prathalingam et al., 2012; Tannenbaum et al., 2012).

The derivation of the hESC line by manual isolation of the ICM and manual passaging would be ideal to avoid contact with xeno-components. Similarly to feeder cell culture, all protocols and reagents should be of GMP-quality, xeno-free, validated, and contamination free. After establishing hESC cell banks, excessive testing needs to be performed to ensure the absence of pathogen contaminants, karyotypic normality, normal growth kinetics, undifferentiated status, and *in vitro* and *in vivo* pluripotency. (Crook et al., 2007; Unger et al., 2008b; Tannenbaum et al., 2012).

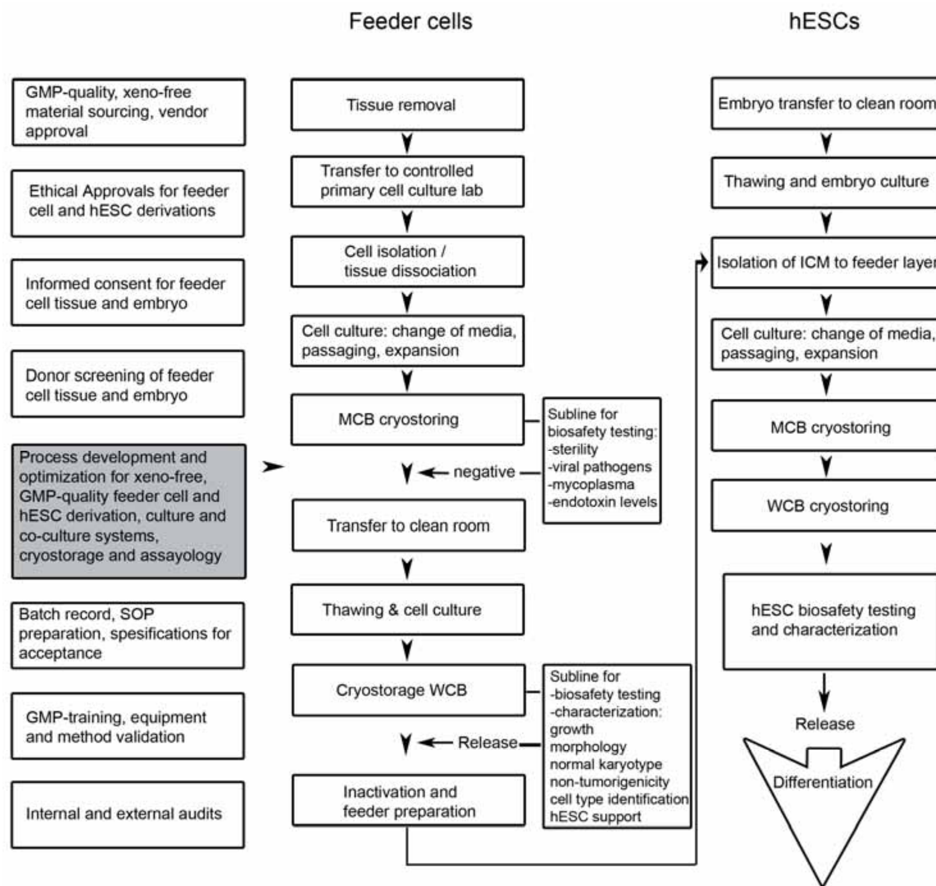


Figure 2. Overview of the processes and requirements for establishment of xeno-free, clinical grade feeder cells and hESC lines. The process development highlighted includes optimization of all the procedures shown for feeder and hESCs cell production.

## 2.4.4 Challenges in human pluripotent stem cell therapies

In addition to the development and optimization of suitable culture conditions and protocols for clinical grade hESCs and eventually hiPSCs, there are many challenges to overcome. The clinical use of hPSCs for cell therapy purposes requires developing robust culture and differentiation systems to produce clinically relevant quantities of undifferentiated cells and differentiated progeny. Enzymatic passaging methods (Hasegawa et al., 2006; Ellerstrom et al., 2007) and rotating 3D bioreactors (Chen et al., 2012) and automated robotic cell culture systems (Terstegge et al., 2007) have been developed to facilitate scaling up hESC culture. Efficient GMP-

compatible differentiation protocols to fully matured and functional cell types, as well as their evaluation and purification methods, are crucial for therapeutic applications. Ideally no undifferentiated cells should be left in the product since even a small number of undifferentiated cells can result in the formation of teratomas in patients (Yamanaka, 2009). Fluorescence-activated cell sorting (FACS) based selection methods for residual undifferentiated hPSCs have been developed to reduce the teratoma-formation potential of heterogeneously differentiated cultures (Tang et al., 2011). Another approach is to transplant precursor or progenitor cells to allow further proliferation and terminal differentiation *in vivo* after transplantation (Puceat and Ballis, 2007).

Regardless of the passaging method, hESCs acquire karyotypic changes during prolonged *in vitro* maintenance that may be correlated to tumorigenic events *in vivo* (Draper et al., 2004b; Mitalipova et al., 2005; Baker et al., 2007). The most recurrent chromosomal changes in hESCs reflect over-expression of genes associated with pluripotency, cell proliferation, and anti-apoptosis, as also seen in common human cancers (Baker et al., 2007). Substantial inter-line and culture-induced epigenetic instability of hESCs have been reported with the majority of the unstable loci uncovered with previous association with tumor phenotypes (Allegrucci et al., 2007). Aberrant miRNA processing also promotes tumorigenesis (Kumar et al., 2007). Directions for the interpretation of such data and grounds for disqualifying a cell line from clinical use need to be established (Vazin and Freed, 2010).

An additional safety issue is related to recipient immune reactions. Histocompatibility is a prerequisite of cell replacement therapy. Some data suggests that hESCs are less susceptible to immune responses due to low level of class I HLA molecule expression or active suppression of the immune response (Li et al., 2004) but contradictory results have also been obtained showing that hESCs can induce immune rejection in immunocompetent rodents (Swijnenburg et al., 2008). Banking of hESC lines has been proposed as a solution. A large but feasible number of carefully selected cell lines could be banked to provide a tissue match for large segments of the population. This approach accepts the use of immunosuppressive medication after transplantation (Carpenter et al., 2009). Estimates of the number of lines needed range from a hundred to several thousands (Taylor et al., 2005; Rao and Auerbach, 2006). Engineering the cells unrecognizable to the immune system

and the use of allogenic hiPSCs are other proposed solutions (Carpenter et al., 2009).

The use of hiPSC technology involves a set of additional problems specific to the transduced cells. The use of oncogenic retroviral vectors to integrate exogenous sequences to the host genome, especially the proto-oncogene c-Myc, poses serious safety concerns for clinical use (Yamanaka and Blau, 2010). Transgenes are largely silenced in hiPSCs, but their reactivation could lead to tumorigenesis (Okita et al., 2007). Since their establishment, many technical improvements have been achieved in the generation of iPSCs with non-viral transduction methods like transposons (Woltjen et al., 2009) or chemical agents like lithium chloride (Wang et al., 2011) to enhance the efficiency of iPSC generation. The forced reprogramming causes genetic and epigenetic changes in the cells and incomplete or aberrant reprogramming may result in an impaired ability to differentiate and may increase the risk of tumorigenicity (Yamanaka and Blau, 2010). The generation, characterization, and safety validation of patient specific clinical-grade iPSC lines will also be very costly (Drews et al., 2012).

## 2.5 *In vitro* differentiation of human pluripotent stem cells to retinal pigment epithelial cells

### 2.5.1 Retinal pigment epithelium

The vertebrate eye has three major components: optic apparatus with cornea, lens and iris; the retina, a photosensitive layer of neural retina containing photoreceptors i.e. rods (night vision) and cones (color vision); and light absorbing, protective layer underneath the photoreceptors containing RPE and choroid (Figure 3.). (Bharti et al., 2011)

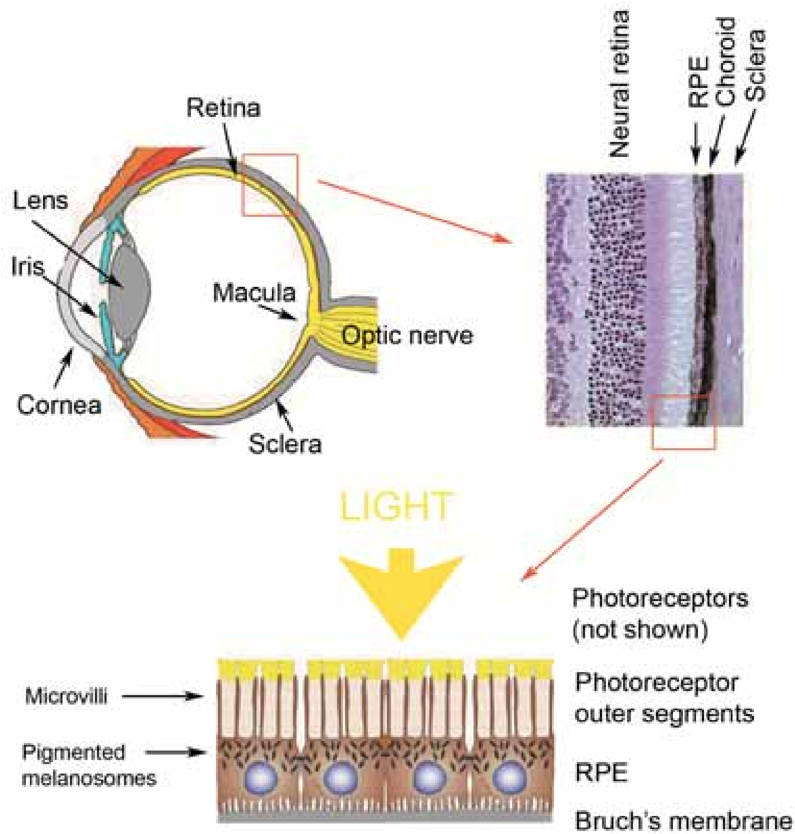


Figure 3. Structure of the human eye and RPE. Figure modified from Bharti et al., 2011.

The RPE is a monolayer of pigmented, polarized and highly specialized epithelium at the outermost layer of the retina. The apical membrane of the RPE faces the photoreceptors with RPE apical microvilli surrounding the photoreceptor outer segments. The basolateral membrane of the RPE faces Bruch's membrane, a specialized basement membrane of the RPE, which separates the RPE from the fenestrated endothelium of the choriocapillaris. (Strauss, 2005) The RPE show characteristic cobblestone-like morphology and pigmentation due to the presence of melanosomes that store melanin pigments. *In vitro* the cells grow in a single monolayer arranged in a regular hexagonal mosaic. (Maminishkis et al., 2006)

Both the RPE and the neural retina are derived from the anterior neural plate during embryogenesis. During neural plate maturation, two bilateral optic primordia are formed to give rise to the right and left eyes. The neural plate rolls into a neural tube and the optic primordia evaginate bilaterally from the diencephalon and give

rise to the optic vesicles. The optic vesicles transform into optic cups, with distinct layers. The inner layer forms the neural retina containing cone and rod photoreceptors, horizontal, bipolar, amacrine, Mueller glial, and ganglion cells, and the outer layer forms the RPE. (Zaghloul et al., 2005)

The cells that compose the early optic vesicles are morphologically and molecularly indistinguishable and co-express transcription factors that are required to initiate eye development: Orthodenticle homeobox-2 (OTX2), Paired box protein-6 (PAX6), Retina and anterior neural fold homeobox (RAX), and SIX homeobox 3 (SIX3). At the optic vesicle stage, the presumptive RPE faces the extraocular mesenchyme while the prospective neural retina faces the surface ectoderm. These surrounding tissues direct the specification of RPE and neural retina respectively. (Fuhrmann et al., 2000; Martinez-Morales et al., 2004) FGFs secreted by the ectoderm direct the development of the adjacent layer to neural fate, in conjunction with the expression of neural retina marker, homeobox-containing transcription factor CHX10, which serves as the earliest specific marker of neural retinal progenitor cells (Rowan et al., 2004). The extraocular mesenchyme secretes activin A and the optic vesicle expresses activin receptors indicating that TGF-ß signaling participates in establishing RPE identity. RPE specification is marked by the expression of transcription factors: Microphthalmia-associated transcription factor (MITF), OTX1/OTX2, and PAX6. It is proposed that PAX6 together with Wnt signaling turn on MITF expression, which, together with OTX proteins, drives the RPE differentiation while PAX6 is down-regulated. In addition, BMP and Sonic hedgehog signaling are involved in the specification of the RPE. RPE differentiation is also linked to the inhibition of cell proliferation corresponding to the withdrawal of RPE progenitor cells from the cell cycle. (Fuhrmann et al., 2000; Martinez-Morales et al., 2004)

The RPE serves many central support functions for the neural retina. It absorbs stray light. It is part of the blood–retina barrier and participates in the control of ion, nutrient, and metabolite transport between photoreceptors and choroid. The RPE functions in the visual cycle of retinal by re-isomerizing all-trans-retinal to 11-cis-retinal by the isomerase, 65 kDa retinal pigment epithelium-specific protein (RPE65). The RPE nurtures the photoreceptors by phagocytosing shed photoreceptor outer segments damaged by light. In addition, the RPE secretes a variety of growth factors to maintain the structural integrity of the choriocapillaris

endothelium and photoreceptors, and immunosuppressive factors participating in the establishment of the immune privilege of the eye. (Strauss, 2005; Bharti et al., 2011)

### 2.5.2 Human pluripotent stem cell therapy for retinal degeneration

One of the most promising clinical applications for hPSC therapies is the correction of loss of vision caused by devastating retinal degenerative diseases like age-related macular degeneration (AMD), Stargardt's macular dystrophy and retinitis pigmentosa. AMD is the leading cause of blindness among elderly people in the Western world, affecting over 50 million people worldwide. The number of people with AMD in Finland is estimated to be approximately 100, 000 and the number is expected to multiply in the near future with the increasing life expectancy (Kaarniranta et al., 2009). AMD is characterized by a progressive loss of central vision attributable to degenerative and neovascular changes in the macula, the area of the central visual field. The progression of AMD leads to the dysfunction and eventually death of the RPE and to impaired photoreceptor function and loss of vision. Approximately 15% of AMD cases are of an atrophic dry form and the rest fall under exudative neovascular, wet form. There is no curative treatment for either type. Anti-neovascular agents, photodynamic therapy and thermal laser treatments are available to alleviate wet AMD. The progression of dry AMD can be delayed with antioxidant therapy, and autologous transplants and macular translocation has also helped some patients. (Gehrs et al., 2006)

Stargardt's macular dystrophy is the most common form of juvenile onset macular degeneration and retinitis pigmentosa is a collection of inherited disorders caused by mutations in a variety of genes expressed by rods. Currently there are no effective treatments for either of these diseases. (Bovolenta and Cisneros, 2009; Rowland et al., 2012)

Cellular replacement therapy to replace damaged RPE and/or photoreceptors is considered the most promising strategy to treat the above-mentioned diseases. A major obstacle with the therapies is the limited supply of cells, making hPSCs with unlimited self-renewal capacity an ideal source of cells for retinal cell therapies (Rowland et al., 2012). In addition, the degree of differentiation can be controlled *in*

*vitro* to ensure optimum safety, identity, purity, and potency before transplantation to patients. The eye, as an immunoprivileged organ with the subretinal space protected by a blood-ocular barrier, is also an attractive candidate for therapeutic hPSC experiments. In addition, the retina can be easily accessed surgically and the transplanted cells directly observed through the clear ocular media (Comyn et al., 2010; Schwartz et al., 2012)

Both hESCs and hiPSCs have been differentiated to RPE cells (Klimanskaya et al., 2004; Buchholz et al., 2009) and photoreceptor progenitor cells (Lamba et al., 2006; Osakada et al., 2009). Moreover, hESCs show capacity to self-organize to optic cup structures and further to form organized, laminated neural retinas in *in vitro* cultures (Nakano et al., 2012). Human PSC-RPE cells have been shown to be capable of photoreceptor rescue and improvement of visual performance in animal models such as the Royal College of Surgeons (RCS) rat model of retinal dystrophy (Lund et al., 2006; Carr et al., 2009a; Idelson et al., 2009; Lu et al., 2009). The first report of a clinical trial with hESC-RPE cells to establish the safety and tolerability of subretinal transplantation in an end disease-stage patient with Stargardt's macular dystrophy and one patient with dry AMD were reported in January, 2012 (Schwartz et al., 2012). Both patients tolerated the transplant well without signs of postoperative inflammation, rejection, or tumorigenicity during the 4-month follow-up. Even some visual improvements were recorded. The hESC line used was a research grade cell line expanded on mEF feeder cells (Schwartz et al., 2012).

The development of efficient, GMP-compatible and xeno-free differentiation methods for hPSC-RPE cells and photoreceptors is needed for clinical transplantation therapies. Other challenges include limited survival upon implantation and the formation of abnormal cell architectures *in vivo*. Attachment of the transplanted cells to Bruch's membrane, survival and integration into the host RPE layer are crucial to the success of a therapeutic strategy. Transplantation of intact cell sheets supported by natural biomaterials or synthetic scaffolds designed to mimic the native *in vivo* ECM surroundings of the RPE, instead of injections of cell suspensions, are thus considered a more suitable transplantation strategy. (Hynes and Lavik, 2010; Rowland et al., 2012)

### 2.5.3 Current *in vitro* differentiation protocols

In 2004, Klimanskaya and co-workers were the first to report differentiation of hESCs to RPE cells. They demonstrated that hESCs can spontaneously differentiate into RPE-like cells when overgrown on mEF feeder cells or without feeder cells as EBs in standard hESC culture medium (ko-SR) but in the absence of bFGF (Klimanskaya et al., 2004). Since then hESC and hiPSC differentiation to RPE cells have been reported by many groups, the differentiation being based on either adherent overgrowth on feeder (usually mEF) cells or EB or neurosphere/rosette formation in suspension. Some of these methods are listed in Table 3.

The pigmentation usually appears within one to eight weeks of culture and after sufficient pigmentation is achieved, pigmented areas are manually selected and seeded to ECM protein coatings for RPE cell enrichment. The differentiation is generally inefficient and it may take months to acquire enough pigmented cells for enrichment. After replating, the hPSC-RPE cells lose pigmentation and acquire fibroblast-like morphology, readily proliferate to confluence and re-differentiate to RPE cell phenotype (Klimanskaya et al., 2004). The transdifferentiation resembles the epithelial-mesenchymal-transition (EMT) process, also described for isolated native RPE cells upon loss of cell-cell contacts (Tamiya et al., 2010).

The spontaneous differentiation methods lead to the generation of multiple cell types and the sequence of events leading to the RPE cell formation is unclear. It is generally accepted that in the absence of inductive cues, the ESCs choose the neural differentiation pathway as a "default" pathway (Smukler et al., 2006). It is possible that the earliest step is a default differentiation step of the neural lineage commitment of neuroectodermal cells or retinal progenitors and the further specification to RPE cells is driven by the surrounding cells in the EBs or the feeder cells (Klimanskaya et al., 2004).

Table 3. Published hPSC-RPE cell differentiation protocols.

| hPSC | Differentiation method: A (cell type) / EB | Key media components: for differentiation (for enrichment) | Publication |
|---|---|---|---|
| hESC | A (mEF) / EB | ko-SR, no bFGF (ko-SR, FBS) | Klimanskaya et al. 2004 |
| hESC | A (mEF) / EB | ko-SR, bFGF, LIF (ko-SR, FBS) | Lund et al. 2006 |
| hESC | A (PA6 cells) | ko-SR, no bFGF | Gong et al. 2008 |
| hESC | EB | ko-SR, no bFGF, Dkk1, Lefty-A | Osakada et al. 2008 |
| hESC | A (mEF) | ko-SR, no bFGF | Vugler et al. 2008 |
| hESC and hiPSC | A (mEF and hFF) | ko-SR, no bFGF (ko-SR, FBS, bFGF) | Buchholz et al. 2009 |
| hESC | A (mEF) | ko-SR, no bFGF | Carr et al. 2009b |
| hiPSC | A (mEF) | ko-SR, no bFGF (N1, taurine, Triiodo thyronin, Hydrocortisone, FBS) | Carr et al. 2009a |
| hiPSC | EB | ko-SR, no bFGF, Dkk1, Lefty-A | Hirami et al. 2009 |
| hESC | EB | ko-SR, no bFGF, NIC, activin A, TGF-ß | Idelson et al. 2009 |
| hESC | EB | MDBK-GM medium, B27 (EGM™-2/MDBK-MM Media) | Lu et al. 2009 |
| hESC and hiPSC | EB | ko-SR, no bFGF, N2, heparin, B27 | Meyer et al. 2009 |
| hESC and hiPSC | EB | ko-SR, no bFGF, CKI-7, SB-431542, ROCKi | Osakada et al. 2009 |
| hESC and hiPSC | A (mEF) | ko-SR, no bFGF | Liao et al. 2010 |
| hESC | EB | B27, ITS, T3, Taurine, HA, Dkk1, Lefty-A, bFGF, RA (FBS) | Nistor et al. 2010 |
| hESC | A (hNSF)<br>A (Matrigel™) | ko-SR, no bFGF (ko-SR, FBS)<br>ko-SR, bFGF, RA, B27, ITS | Harness et al. 2011 |
| hiPSC | EB | ko-SR, no bFGF, NIC, activin A, SB-431542 (EpiCM medium) | Kokkinaki et al. 2011 |
| hESC and hiPSC | EB | ko-SR, no bFGF / RegES, no bFGF, no retinol | Vaajasaari et al. 2011 |
| hESC | EB | ko-SR, N2, B27, Noggin, Dkk1, IGF, (ko-SR, bFG, FBS) | Zhu et al. 2011 |
| hESC | EB (neural rosettes) | ko-SR, N2, B27, bFGF | Park et al. 2012 |
| hiPSC | neural rosettes | ko-SR, N2, Noggin, bFGF, SB-431542, RA, Shh | Zahabi et al. 2012 |

Abbreviations: A, adherent on feeder cells; EB, embryoid body differentiation in suspension; PA6 cells, mouse stromal cells; hNSF, human neonatal skin derived fibroblasts; N1, culture media supplement containing insulin, transferrin, sodium selenite, putrescine, and progesterone (Sigma-Aldrich); MDBK-GM, GMP-quality complete culture medium (Sigma-Aldrich); EGM™-2, endothelial cell growth medium (Lonza); MDBK-MM Medium, Madin-Darby Bovine Kidney Maintenance Medium, protein-free (Sigma-Aldrich); T3, triiodothyronine; N2, chemically defined neuronal cell supplement (Life Technologies); B27, serum-free neuronal cell supplement (Life Technologies); EpiCM medium, Epithelial Cell Medium (ScienCell Research Laboratories); RegES, xeno-free hESC culture medium; Shh, sonic hedgehog. Other abbreviations are presented on page 13.

Various growth factors and small molecules have been used to direct and enhance hPSC differentiation to neuroectoderm and then further to retinal precursor and RPE fate in suspension cultures. Wnt antagonist Dickkopf-1 (Dkk1) combined to nodal antagonist Lefty-A (Osakada et al., 2008) and stable small molecule inhibitors of the same pathways, CKI-7 and SB431542 respectively (Osakada et al., 2009) have been used to induce retinal progenitor cell differentiation. Nicotamide (vitamin B3, NIC, which is a potent neuroprotector and cell survival factor) has been shown to stimulate RPE cell differentiation during the first four weeks of culture, when neural and retinal specification occurs (Idelson et al., 2009). After initial neural

differentiation, the addition of TGF-ß or high levels of activin A has been used to enhance RPE cell differentiation (Idelson et al., 2009; Kokkinaki et al., 2011; Meyer et al., 2011). IGF-1 in combination with noggin and Dkk1 can promote neural precursor or retinal progenitor identity before further induction to neural retina or RPE direction (Lamba et al., 2006; Lamba and Reh, 2011; Zhu et al., 2011). Recently hPSC-RPE cell differentiation using xeno-free and defined RegES culture medium was reported (Vaajasaari et al., 2011).

Although essentially similar, there are reports of important differences between hESC and hiPSC-derived RPE cells. Some studies have reported hiPSCs to be inferior in differentiation capacity (Buchholz et al., 2009; Meyer et al., 2009) and to undergo accelerated senescence, raising concerns for clinical applications (Kokkinaki et al., 2011). Recent global gene expression studies have also revealed differential gene expression in hPSC-RPE cells compared to fetal RPE, suggesting that hiPSC-RPE cells especially are in a relatively immature differentiation stage (Liao et al., 2010). There is a great need for further studies on the differentiation process to identify inducers and develop robust, well characterized, xeno-free, as well as GMP-compatible differentiation methods to generate hPSC-RPE cells which closely resemble native RPE.

### 2.5.4 Characterization of human pluripotent stem cell-derived retinal pigment epithelial cells

Human PSC-RPE cells show typical pigmentation and morphology similar to their native counterparts. They express genes and proteins specific for RPE, including transcription factors MITF and OTX2, membrane associated proteins bestrophin, and tight junction protein zona occludens 1 (ZO-1), proteins involved in the retinal visual cycle like cellular retinaldehyde-binding protein (CRALBP) and RPE65, tyrosinase and premelanosome protein (PMEL), which functions in pigment synthesis, proteins involved in phagocytosis, like the integrin αV subunit and Mer Tyrosine Kinase (MERTK) (Lund et al., 2006; Vugler et al., 2008; Buchholz et al., 2009; Carr et al., 2009b; Liao et al., 2010; Vaajasaari et al., 2011; Cho et al., 2012). Human PSC-RPE cells also show a gene expression signature similar inpart to native RPE (Liao et al., 2010; Lamba and Reh, 2011) and dynamic regulation of

specific miRNA subsets associated with the differentiation process (Li WB et al., 2012).

Mature hESC-RPE cells show polarized apical and basal features and barrier function with increasing transepithelial electric resistance (TER) and impedance and decreasing permeability, measured by electrical impedance spectroscopy (Savolainen et al., 2011; Vaajasaari et al., 2011; Zhu et al., 2011; Onnela et al., 2012). Polarized hPSC-RPE cells show localization of proteins e.g. apical localization of $Na^+/K^+$ATPase and basolateral localization of bestrophin and polarized secretion of vascular endothelial growth factor (VEGF) to the basolateral side (Kokkinaki et al., 2011; Vaajasaari et al., 2011). Polarized hESC-RPE cells show prominent expression of pigment epithelium-derived factor (PEDF) in apical cytoplasm and increase in secretion of PEDF into the medium compared with non-polarized culture (Zhu et al., 2011).

The functionality of hPSC-RPE cells is shown by their ability to phagocytose latex beads or more specifically rod outer segments (Klimanskaya et al., 2004; Buchholz et al., 2009; Liao et al., 2010) and rescue visual function in the RCS rat model(Lund et al., 2006; Vugler et al., 2008).

# 3. Aims of the study

The general objective of the thesis was to test, develop and optimize defined, xeno-free and GMP-compatible culture methods for hESCs. Special focus was in finding alternatives to the current supporting feeder cell system from feeder-independent culture methods and xeno-free feeder cells. In addition, the function of the feeder cells in supporting hESC self-renewal and pluripotency, and the effect of the feeder cells on the differentiation of RPE cells from hPSCs were studied. The specific aims of the substudies are outlined below:

1. To test and evaluate different xeno-free culture media, especially human serum-containing culture media for the *in vitro* culture of undifferentiated, pluripotent hESCs. (I)

2. To test and evaluate different feeder-independent culture systems for undifferentiated, pluripotent hESCs based on the use of ECM preparations and selected biomaterials as a culture base in combination with specific media. (II)

3. To test and optimize protocols for xeno-free and eventually GMP-compatible feeder cell production with respect to:

- Derivation of fibroblast feeder cells from dermal tissue and testing the cells for hESC support (III)
- Xeno-free fibroblast culture medium based on human serum supplementation (III)
- Optimizing fibroblast culture, cryopreservation and feeder cell preparation protocols (III)

4. To study the role of the supporting feeder cells for:

- Undifferentiated, pluripotent hESC culture (III)
- Differentiation of hPSCs to RPE cells (IV)

# 4. Materials and methods

## 4.1 Cell lines and culture (I-IV)

### 4.1.1 Human embryonic stem cell culture (I-IV)

The hESC lines HS181 (46, XX), HS237 (46, XX; 46, X, abnormal X), HS293 (46, XY), HS306 (46, XY) and HS401 (46, XY) were derived at the Fetility Unit of Karolinska University Hospital, Huddinge (Karolinska Institute, Stockholm, Sweden). The derivation and characterization of the cell lines have been published previously (Hovatta et al., 2003; Inzunza et al., 2005). The hESC lines Regea06/015 (46, XY), Regea07/046 (46, XX), Regea08/017 (46, XX), and Regea06/040 (46, XX) were derived at The Institute of Biomedical Technology (IBT) (formerly Regea – Institute for Regenerative Medicine), University of Tampere, Finland. The derivation and characterization of Regea06/015 and Regea07/046 hESC lines has been published previously in (Rajala et al., 2010). Regea08/017 and Regea06/040 are derived and characterized as published in (Skottman, 2010).

Prior to beginning of the experiments, the hESCs were cultured on mitotically inactivated hFF feeder cells (CRL-2429, ATCC). A confluent layer of inactivated feeder cells was seeded on 35 mm tissue culture dish (BD Falcon, BD Biosciences, NJ, USA) and allowed to attach overnight. Undifferentiated hESC colonies were plated directly on to the feeder cells in standard hESC culture medium (hESC medium). The hESC medium consisted of Knockout™-Dulbecco's modified Eagle's medium (ko-DMEM) supplemented with 20% Knockout Serum Replacement (ko-SR), 2mM GlutaMAX-I (all from Life Technologies, Paisley, UK), 1 % MEM Eagle Non-Essential Amino acid solution (NEAA, Cambrex Bio Science, Walkersville, MD,USA), 50 U/ml penicillin/streptomycin (Cambrex Bio Science), 0.1mM ß-mercaptoethanol (Life Technologies) and 8 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA). The cells were cultured at 37ºC, 5% $CO_2$ and monitored daily by microscoping. The hESC medium was changed 5-6 times a week

and hESCs were manually passaged once a week. Manual passaging was performed by cutting undifferentiated colonies with scalpel and transferring colony pieces to new feeder layers.

All hESC lines were regularly characterized for the expression of proteins associated with the undifferentiated, pluripotent hESC state (Nanog, Oct-3/4, SSEA-3, SSEA-4, TRA-1-81, and TRA-1-60) by immunofluorescence. The pluripotency was analysed *in vitro* by embryoid body formation followed by RT-PCR analysis for markers of the three embryonic germ layers. The karyotypic stability was analysed by G-banding of metaphase cells at Medix Laboratories (Espoo, Finland).

### 4.1.2 Human induced pluripotent stem cell culture (IV)

The hiPSC line FiPS5-7 was derived and characterized by Professor Otonkoski's research group at University of Helsinki, Finland. The FiPS5-7 was generated from human fibroblasts using four factors: Oct-3/4, Sox-2, Nanog, and Lin-28 (Rajala et al., 2010). Transgene silencing was confirmed with qPCR (Hussein et al., 2011). The hiPSC line was initially derived on and cultured on mitotically inactivated mEF feeder cells. At passage level 25 the cell line was transferred to hFF feeder cells and cultured thereafter as described above for hESC lines.

### 4.1.3 Fibroblast cell cultures (I-IV)

#### *4.1.3.1 Human foreskin fibroblast culture (I-IV)*

CRL-2429 hFF cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were purchased as frozen stock at passage level 4. C-12300 (PromoCell GmbH, Heidelberg Germany) hFF cells were purchased as frozen stock at passage level 2. The cells were routinely cultured in FBS-medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and 25 mM HEPES, supplemented with 10% FBS (both from Life Technologies) and 0.5% penicillin/streptomycin. For xeno-free culture in HS-medium, the FBS was replaced with human serum (Type AB, PAA Laboratories GmbH Pasching, Austria).

For preparation of feeder cells the fibroblasts were inactivated by gamma irradiation (40 Gy) or with 10 µg/ml Mitomycin C (Sigma-Aldrich, St Louis, MO, USA) for 3 hours at 37ºC. Inactivated cells (3.65 x $10^4$ cells/$cm^2$) were plated to culture dishes (Corning Incorporated, NY, or Nalge Nunc International, Rochester, NY, USA). Adaptation to serum-free culture was performed by gradually changing to hESC medium.

#### *4.1.3.2 Human dermal fibroblast culture (III)*

Five hDF cell lines: hDF 001/06, hDF 002/06, hDF 003/06, hDF 001/07, hDF 001/08 were derived from dermal tissues obtained from children under the age of 1 year in context of surgery from Tampere University Hospital, Tampere, Finland. The fibroblast cell lines were derived as explant cultures similarly to a previously published protocol (Takashima, 2001). The tissue pieces were extensively washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Group Ltd., Basel, Switzerland). Subcutaneous tissue and epidermis were manually removed and dermis cut into 1 $mm^2$ pieces, placed on tissue culture dishes and covered with sterile coverslips (Menzel-Gläser, Braunschweig, Germany). HS-medium was used for derivation and culture and changed every 3-4 days. The hDF 003/06 cells were simultaneously derived to both HS-and FBS-media. The outgrowth of fibroblasts was monitored by daily microscopic inspections.with Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe). Upon confluency the fibroblasts were harvested and subcultured with TrypLE Select (Life Technologies) and cryopreserved at early passages in HS-medium supplemented with 5-10% dimethyl sulfoxide (DMSO). The feeder cells were prepared as described above.

#### *4.1.3.3 Mouse embryonic fibroblast culture (III-IV)*

Mouse EF cells used for laminin chain immunoprecipitation experiments were derived from p.c. ICR fetuses and cultured in 10% fetal bovine serum and GlutaMAX-I in DMEM (all from Life Technologies). The cells were derived and cultured at the Biomedicum Stem Cell Center, University of Helsinki. (III)

Mouse EF cells (P-MEF, EmbryoMax®) for hPSC-RPE cell differentiation experiments were purchased as frozen stock from Millipore (Billerica, MA, USA) and cultured in sterile filtered medium consisting of ko-DMEM supplemented with 10% FBS (PAA Laboratories GmbH) and 1% GlutaMAX-I. Cell cultureware for mEFs were pre-coated with 0.1% porcine gelatin (Sigma-Aldrich) for at least 1 h at room temperature (RT). The mEF cells were cryopreserved in FBS supplemented with 10% DMSO. (IV)

#### *4.1.3.4 Collection of conditioned media (II, IV)*

For feeder-independent cultures, the hFF conditioned hESC medium (hESC-CM) was collected by incubating standard hESC culture medium overnight on irradiated hFF feeder plates. An additional 8 ng/ml bFGF was freshly added to the hESC-CM before using in feeder-independent hESCs cultures, except for the biomaterial testing. (II)

For RPE cell differentiation experiments, RPEbasic medium (see Chapter 4.1.7) was conditioned on irradiated hFF and mEF feeder cells cultured on 0.1% gelatin. For a period of 10 days, 2 ml/cm$^2$ of RPEbasic was collected daily from the culture dishes and replaced with equal amount of fresh medium. Pooled hFF-CM and mEF-CM media were stored at -70°C until used for differentiation experiments. Four different batches of CM were similarly prepared for both fibroblast types. (IV)

### 4.1.4 Xeno-free human embryonic stem cell culture media (I)

In addition to standard hESC medium three commercial culture media: X-Vivo™ 10 and X-Vivo™ 20 (both from Cambrex BioScience) and previously published TeSR1 medium (Ludwig et al., 2006a) with modifications were tested in supporting undifferentiated hESC culture. Also, five commercially available serum replacements: Lipumin™; SerEx™ (both from PAA Laboratories GmbH); SR3 (Sigma–Aldrich); serum substitute supplement (SSS™) (Irvine Scientific, Santa Ana, CA, USA) and Plasmanate® (Bayer Healthcare, West Haven, CT, USA) were tested. All serum replacements were tested as 10% and 20% in ko-DMEM, except for Plasmanate® that was tested as 20% and 40%. Also, heat inactivated (57ºC, 30

min) HS (H1388) (Sigma–Aldrich) was tested. The HS containing media were additionally supplemented with 50 mg/l L-ascorbic acid 2-phosphate (Sigma–Aldrich). For concentrations tested, media compositions, hESC lines and passages used, see Table I in Article I.

Irradiated hFF feeder cells were used for all cultures. The hESCs were adapted to the test media by gradually increasing the proportion of the test medium to that of hESC culture medium. All media were tested with at least two different hESC lines and tests repeated for the media that failed to maintain undifferentiated hESC culture.

### 4.1.5 Feeder-independent culture methods (I, II)

Different published feeder cell-independent culture methods, and modifications of these, as well as culture on selected biomaterials were evaluated for long-term, undifferentiated hESC culture. The matrix and media combinations tested and the publications they were based on are summarized in Table 4. For hESC lines and passages used, see Table I in Article I and Table I in Article II.

Modified hESC medium (mhESC) consisted of same reagents as the standard hESC medium with the modifications of 15% ko-SR, 0.12 ng/ml TGF-ß (Sigma–Aldrich) and 4 ng/ml bFGF. Chemically defined medium (CDM) consisted of IMDM and F12 with Glutamax I (Life Technologies) basal media (1:1), supplemented with 5 mg/ml human serum albumin (HSA), 450 mM monothioglycerol, 15 μg/ml human holo-transferrin (all from Sigma-Aldrich), 1% chemically defined lipid concentrate, 7 μg/ml human recombinant insulin (both from Life Technologies), 10 ng/ml bFGF, and 12 ng/ml activin A (both from R&D Systems).

Sera coatings were prepared with 10% heat inactivated HS (PAA Laboratories GmbH) or FBS (Life Technologies) in IMDM and 0.5% penicillin/streptomycin at 37ºC, 1-7 days. 100% HS coating tested with hESC medium and hESC-CM was prepared with undiluted HS (H1388, Sigma-Aldrich) at 37ºC overnight.

Xeno-free TeSR1 medium was tested as two versions. Version 1 was identical to that tested for hESC culture on feeder cells (Chapter 4.1.4), except with the modification of 100 ng/ml bFGF. Version 2 was similar to version 1. but with

modifications of 12.9 mg/ml HSA, 22.8 µg/ml insulin, 10.4 µg/ml human holotransferrin and 45 mg/l L-ascorbic acid 2-phosphate. The mTeSR™1 medium was purchased from STEMCELL Technologies (Grenoble, France) and handled according to the manufacturer's instructions with 0.5% penicillin/streptomycin added.

Xeno-free CELLstart™ matrix (1:30 dilution, at 37ºC for 1 hour) was tested in combination with serum-free but xeno-compound containing StemPro® hESC SFM medium (Both from Life Technologies). StemPro® hESC SFM consisted of DMEM/F-12+Glutamax supplemented with 1x StemPro® hESC SFM supplement, 1.8% BSA (all from Life Technologies), 8 ng/ml bFGF, 0.1 mM ß-mercaptoethanol and 0.5% penicillin/streptomycin. Regea 06/015 hESC line at passage level 82 was used for testing.

Table 4. Feeder-independent hESC culture methods tested.

| Based on publication by | Matrix/Biomaterial | Media | Tested in Article |
|---|---|---|---|
| NP | Ti (Vivoxid Ltd., Turku, Finland) | hESC medium/ hESC-CM | II |
| | $TiO_2$ (Vivoxid Ltd.) | | |
| | $ZrO_2$ (Turku Biomaterials Centre, University of Turku, Finland) | | |
| | PDTEC (Institute of Biomaterials, Tampere University of Technology, Finland) | | |
| | PLDLA (Institute of Biomaterials) | | |
| Amit et al., 2004; Amit and Itskovitz-Eldor, 2006 | 5 µg/cm$^2$ human fibronectin (Sigma–Aldrich) | mhESC | II |
| Vallier et al. 2005 | 5-20 µg/cm$^2$ human fibronectin | CDM | II |
| Amit et al., 2004; Amit and Itskovitz-Eldor, 2006 | BD BioCoat™ Fibronectin cultureware (BD Biosciences) | mhESC | II |
| Vallier et al. 2005 | | CDM | II |
| Vallier et al. 2005 | 10% FBS coating (Life Technologies) | CDM | II |
| NP | 10% HS coating (PAA Laboratories GmbH) | CDM | II |
| Stojkovic et al. 2005b | 100% HS-coating (Sigma–Aldrich) | hESC medium/ hESC-CM | II |
| Ludwig et al. 2006a | hECM mix: 10 µg/cm$^2$ collagen IV 0.2 µg/cm$^2$ vitronectin 5 µg/cm$^2$ fibronectin 5 µg/cm$^2$ laminin (Sigma–Aldrich) | TeSR1 version 1. | I |
| | | TeSR1 version 2. | II |
| NP | | hESC medium / hESC-CM | II |
| NP | | 20% HS-medium +80 ng/ml bFGF | I |
| NP | hESC-qualified Matrigel™ (Life Technologies) | hESC medium/ hESC-CM | II |
| Ludwig et al. 2006b; commercial | | mTeSR™1 (STEMCELL Technologies) | II |
| Wang et al. 2007; commercial | 1:30 CELLstart™ (Life Technologies) | StemPro® hESC SFM (Life Technologies) | * |

Abbreviations: NP, not published previously; hESC-CM, hFF-conditioned hESC medium; TeSR1, xeno-free hESC medium; mTeSR™1; xeno-compound containing modified TeSR1 medium; CELLstart™, xeno-free cell culture matrix; StemPro® hESC SFM; serum-free but xeno-compound containing hESC culture medium. Other abbreviations are presented on page 13.
*Published in Ojala, 2009.

The hESCs were passaged manually to the feeder-independent culture systems and thereafter either manually or with a combination of manual and enzymatic techniques using 0.5-1 mg/ml dispase or 1-5 mg/ml collagenase IV (both from Life Technologies) every 3-7 days when the colonies reached an appropriate size without excessive differentiation. Culture media were changed 6 times a week.

## 4.1.6 Xeno-free fibroblast culture media (III)

Different human sera, sera concentrations, additives to culture media and serum-free culture media listed in Table 5 were tested for fibroblast culture. The media were tested with either CRL-2429 or C-12300 hFF cells or with in-house derived hDF cell lines. Fibroblasts were gradually adapted to each media and cells in exponential growth phase were used for testing. The fibroblast growth and morphology were monitored daily with microscope and the cells were passaged with TrypLE Select upon confluency.

Table 5. Human sera and serum-free media tested for fibroblast culture.

| **Serum*** | **Type** | **Manufacturer** |
|---|---|---|
| 10% FBS (control) | Heat inactivated | Life Technologies |
| 5-20% HS | Human serum from clotted male whole blood | Sigma-Aldrich |
| | Human serum AB | Paa Laboratories |
| | Human serum AB | Lonza |
| | Human serum AB, heat inactivated | Lonza |
| | Human serum AB, male only | Lonza |
| | Human serum AB | Equitech Bio Inc. |
| | Human serum charcoal stripped, defibrinated, delipized | Equitech Bio Inc. |
| **Serum*/replacement* /medium** | **Additives/Note** | **Manufacturer** |
| 5% HS | 8 ng/ml bFGF, 5 ng/ml TGF-ß1,<br>50 mg/l L-ascorbic acid (Sigma-Aldrich) | Sigma-Aldrich |
| 5% HS | 10 ng/ml activin A (R&D Systems) | Sigma-Aldrich |
| 5% HS | 1 ng/ml TGF-ß1 | Paa Laboratories |
| 5% HS | 1 ng/ml bFGF | Paa Laboratories |
| 5% HS | 50 mg/l L-ascorbic acid | Paa Laboratories |
| 10% ko-SR | 1% NEAA | Life Technologies |
| 10% RegES | 1% NEAA, 6 mg/ml HSA (Sigma-Aldrich)<br>10 µg/ml insulin (Life Technologies) | Regea research product, xeno-free |
| 10% RegES | 1% NEAA, 12 mg/ml HSA, 20 µg/ml insulin,<br>8 ng/ml bFGF, 5 ng/ml TGF-ß1(Sigma-Aldrich)<br>1:100 defined lipid concentrate (Life Technologies) | Regea research product, xeno-free |
| 20% Lipumin™ 10X | 1% NEAA | Paa Laboratories |
| 20% SerEX 10X | 1% NEAA | Paa Laboratories |
| 20% Serum Substitute Supplement (SSS) | 1% NEAA | Irvine Scientific |
| FibroLife® Serum-Free Cell Culture Medium | With/without 2% HS | Lifeline Cell Technology |
| StemPro® MSC SFM | Serum-free culture medium for MSCs | Life Technologies |

*Basal medium: IMDM supplemented with 0.5% penicillin/streptomycin. Abbreviations are presented on page 13.

### 4.1.7 Differentiation culture of human pluripotent stem cells to retinal pigment epithelial cells (IV)

Regea06/040 hESCs and FiPS5-7 hiPSCs were differentiated to RPE cells in suspension cultures in three different media: RPEbasic medium, RPEbasic conditioned on hFF feeder cells (hFF-CM) or RPEbasic conditioned on mEF feeder cells (mEF-CM). The RPEbasic differentiation medium was identical to standard hESC culture medium, with the modifications of 15% ko-SR and no bFGF. For activin A supplementation, 10 ng/ml activin A (Peprotech, NJ, USA) was added to RPEbasic. The pigmented cells were selectively replated to adherent cultures on collagen IV. For details, see Article IV.

## 4.2 Characterization of undifferentiated human embryonic stem cells (I-III)

### 4.2.1 Colony morphology (I-III)

Undifferentiated hESC state was primarily characterized by typical colony morphology. Spontaneous differentiation led to loss of smooth colony surface and defined edges (Figure 4). In feeder-independent cultures cells differentiated to fibroblast-like cells (hESC-df), first at the colony borders and then progressively throughout the colony (Figure 4E and F).

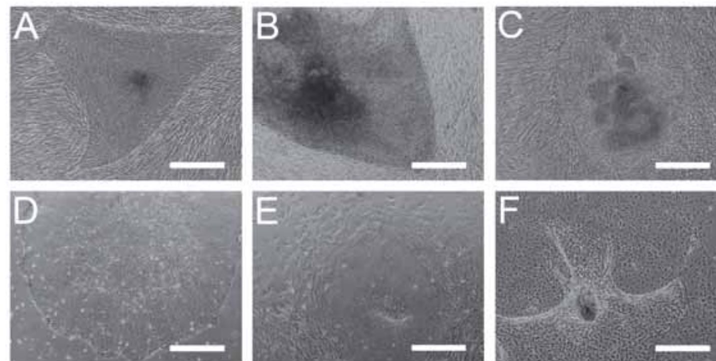


Figure 4. Typical hESC colony morphologies. A) Undifferentiated, B) partly differentiated and C) completely differentiated hESC colony on hFF feeder cells. D) Undifferentiated, F) partly and G) completely differentiated hESC colony in feeder-independent culture. Scale bars 500 µm for A-C and 200 µm for D-F.

### 4.2.2 Q-PCR (II-III)

Human ESCs were analysed for the relative expression of pluripotency genes: Oct-3/4, Nanog, DNMTB3 and TDGF1, with relative quantitative real-time polymerase chain reaction (q-PCR). Regea06/015 cells cultured on Matrigel™ in mTeSR™1 for 10, 21, and 32 passages were analysed (II). Regea07/046 hESCs cultured on hFF CRL-2429 and hDF 001/08 feeder cells for 1-6 passages (until complete hESC differentiation on hDF 001/08) were analysed (III).

All hESC colonies without selection were collected for analysis. Total RNA was extracted with Qiagen RNeasy Plus Mini kit and RNA concentration and quality were assessed with NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was reverse-transcribed to cDNA with Sensicript Reverse Transciription kit (Qiagen) (II) or High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) (III). FAM-labeled TaqMan Gene Expression Assays (Applied Biosystems) were used for PCR reactions. All samples and no template controls were analyzed as three replicates and Ct values were determined for every reaction. Relative quantification as fold change compared to reference level, was calculated with the -2ΔΔCt method (Livak and Schmittgen, 2001). The data was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as endogenous control. The expression levels of hESCs cultured on hFF cells in hESC culture medium were used as reference level (fold change = 1). For fold change values greater than one, fold regulation = fold change. For fold change values less than one, fold regulation was calculated as -1/(fold change). Standard deviations of each set of technical replicates were presented as error bars. For details, see Articles II and III.

### 4.2.3 Immunofluorescence (I-III)

For immunofluorescence the hESC colonies were fixed with 4% paraformaldehyde (PFA) (Sigma–Aldrich), for 20 min at RT. The cells were permeabilized and blocked with 0.1% Triton X-100, 1% BSA and 10% normal donkey serum (all from Sigma–Aldrich) in DPBS for 45 min at RT. Cells were labeled with primary antibodies overnight at 4ºC, diluted in 0.1% Triton X-100, 1% BSA and 1% normal donkey serum in DPBS. Secondary antibodies diluted in 1% BSA in DPBS were

incubated for 1 hour in the dark at RT. DPBS was used for washes and phosphate buffer (Sigma–Aldrich) for the final wash. Vectashield mounting medium containing 40,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) was used for nuclei counterstaining. The cells were imaged with Olympus IX51 phase contrast microscope with fluorescence optics and Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan). For negative control stainings the primary antibodies were omitted. Antibodies and dilutions used are listed in Table 6 and Table 7. For details see Articles I-III.

Table 6. Primary antibodies used for immunostaining of hESCs.

| Primary Ab | Source animal | Manufacturer | Dilution | Article |
|---|---|---|---|---|
| Nanog | Goat IgG | Santa Cruz Biotechnology, Santa Cruz, CA, USA | 1:200 | I |
| Nanog | Goat IgG | R&D Systems | 1:200 | II, III |
| SSEA-1 | Mouse IgM | Santa Cruz Biotechnology | 1:200 | I, II |
| Oct-3/4 | Goat IgG | R&D Systems | 1:300 | I, II |
| SSEA-3 | Rat IgM | Novus Biologicals, Littleton, CO, USA | 1:300 | II |
| SSEA-4 | Mouse IgG | Santa Cruz Biotechnology | 1:200 | I, II |
| TRA-1-81 | Mouse IgM | Santa Cruz Biotechnology | 1:200 | II |
| TRA-1-60 | Mouse IgM | Millipore, Billerica, MA, USA | 1:200 | II |

Table 7. Secondary antibodies used for immunostaining of hESCs.

| Secondary Ab | Conjugate | Manufacturer | Dilution | Article |
|---|---|---|---|---|
| Donkey anti-mouse IgM | Rhodamine Red | Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK | 1:400 | I |
| Donkey anti-goat IgG | Alexa Fluor 488 | Molecular Probes, Life Technologies | 1:800 | I, II |
| Donkey anti-goat IgG | Alexa Fluor 568 | Molecular Probes | 1:800 | II,III |
| Goat anti-mouse IgM | Alexa Fluor 568 | Molecular Probes | 1:800 | II |
| Goat anti-mouse IgG | Alexa Fluor 568 | Molecular Probes | 1:800 | II |
| Goat anti-mouse IgM | Alexa Fluor 488 | Molecular Probes | 1:800 | II |
| Anti-rat IgM | FITC | Novus Biologicals | 1:800 | II |

### 4.2.4 Flow cytometry (I-III)

HS237 hESCs cultured in 20% HS containing culture medium after 11 passages and in hESC medium (control) were analyzed for SSEA-4 expression (I). HS401 hESCs cultured for 28 passages and Regea06/015 cultured for 23 passages on Matrigel™ in mTeSR™1 medium were analyzed for SSEA-4 and TRA-1-81 expression (II). Regea07/046 and Regea08/017 hESCs cultured on hFF CRL-2429 and hDF 001/08 feeder cells were analyzed for TRA-1-81 and Oct-3/4 expression over 10 passage

culture period. As TGF-ß1 growth factor was added to hDF 001/08 culture, the cells were analyzed for TRA-1-81 expression during passages 2-6 (III).

All hESC colonies without selection were collected for analysis. The hESCs were dissociated to single cells with TrypLE Select, collected by centrifugation and resuspended to buffer. Cells (0.1 x $10^6$) were probed with either primary antibodies followed by secondary antibodies, or with directly conjugated flow cytometry antibodies. For labeling with intracellular Oct-3/4, cells were fixed with 4% PFA for 10 min at RT prior to the labeling. After each step the cells were washed with buffer and collected by centrifugation. The cells were analyzed either directly or after fixation with 1% formaldehyde. BD FACSAria (BD Biosciences) was used for analysis and the acquisition was set to 1-2 x $10^4$ events per sample. The data was analyzed using FACSDiva Software version 4.1.2 (BD Biosciences). Antibodies and buffers used are listed in Table 8. Unlabelled cells were used as negative controls. Primary antibodies were omitted for controlling unspesific binding of secondary antibodies and appropriate isotype controls were used.

Table 8. Reagents used for flow cytometry analyses.

| Article | Antibodies | Manufacturer | Dilution | Buffer | Incubation |
|---|---|---|---|---|---|
| I | mouse anti-human SSEA-4 | Santa Cruz Biotechnology | 1:500 | Buffer I: 2% FBS, 0.01% $NaN_3$ in DPBS<br>Buffer II: 0.01% $NaN_3$ in DPBS | 15 min, 4ºC |
| | PE-conjugated goat anti-mouse IgG | Molecular Probes, Life Technologies | 1:500 | | 15 min, 4ºC, in the dark |
| II | PE-conjugated mouse anti-human SSEA-4 antibody | R&D systems | 1:200 | 0.5% BSA in DPBS | 45 min, 4ºC, in the dark |
| | Mouse anti-human TRA-1-81 | Santa Cruz Biotechnology | 1:200 | 2% FBS in DPBS | 15-30 min, 4ºC |
| | PE-conjugated anti-mouse IgM | Molecular Probes, Life Technologies | 1:500 | | 20 min, 4ºC, in the dark |
| III | FITC conjugated mouse anti-human TRA-1-81 | BD Biosciences | 8 µl | 0.5% BSA, 0.01% $NaN_3$ in DPBS | 30 min, 4ºC, in the dark |
| | PE-conjugated anti-human Oct-3/4 | R&D systems | 10 µl | 0.1% Saponin, 0.05% NaN3 in DPBS | 30 min, RT, in the dark |

Abbreviations: NaN3, sodium azide. Other abbreviations are presented on page 13. FBS from Life Technologies, $NaN_3$, BSA and Saponin from Sigma-Aldrich.

### 4.2.5 Cell proliferation assay (III)

Cell proliferation of Regea08/017 hESCs cultured on hFF CRL-2429 and hDF 001/08 feeder cells was determined using colorimetric immunoassay (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Mannheim, Germany) based on the measurement of BrdU incorporation during DNA synthesis. For details, see Article III.

### 4.2.6 Karyotyping (II)

Karyotype analysis was performed on HS401 hESCs after 24 passages and for Regea06/015 after 35 passages on Matrigel™ matrix in mTeSR™1 medium, and for Regea06/015 after 24 passages on CELLstart™ matrix in StemPro® hESC SFM medium. For karyotyping the hESCs were transferred to hFF feeder cell layers in hESC culture medium for 1-4 passages prior the analysis. A cytogenetic analysis of 20 metaphase cells was performed using G-banding at Medix Laboratories Ltd, Espoo, Finland.

## 4.3 Fibroblast analyses (III, IV)

### 4.3.1 Liquid chromatography–mass spectrometry for Neu5Gc analysis (III)

The presence of sialic acid Neu5Gc was detected from C-12300 cells cultured to passage level 7 with HS-medium and passage level 15 with FBS-medium, and from hDF 003/06 cells derived to and cultured with HS- and FBS-media to passage level 3. Also, hDF 003/06 cells initially derived to FBS-medium but transferred to HS-medium at the second passage and cultured for 13 days in HS-medium were analysed. Approximately 1 x $10^6$ cells per sample were used for measurements. The Neu5Gc measurements were performed with liquid chromatography–mass spectrometry (HPLC-MS) method by Professor Seppo Auriola's research group at University of Eastern Finland.

### 4.3.2 Quantitative PCR analyses of extracellular matrix (III)

The ECM gene expression profiles were compared with q-PCR array from three biological replicates of irradiated hFF CRL-2429 (passages 9, 10 and 11) and hDF001/08 (passages 3, 4 and 5) feeder cells. Human Extracellular Matrix and Adhesion Molecules – $RT^2$ Profiler$^{TM}$ PCR Array (PAHS-013A, SABiosciences, MD, USA) was used and data was analyzed with $RT^2$ Profiler™ PCR Array Data Analysis software (http://www.sabiosciences.com/pcr/arrayanalysis.php). The samples were additionally analyzed for laminin chain α4, α5, γ2 and laminin receptor B-CAM expression with TaqMan Gene Expression Assays. Fold change and fold regulation values were calculated as described above in section 4.2.2. The expression levels of hFF were used as reference level for each gene. For details, see Article III.

### 4.3.3 Immunoanalyses of laminin alpha-5 chain (III)

Production of laminin α5 chain by the hFF, mEF and hDF feeder cell lines was studied by immunoprecipitation from the culture supernatant. Laminin α5 expression by the hFF and hDF cell lines was additionally studied with immunofluorescence staining. For details, see Article III.

### 4.3.4 Enzyme-linked immunosorbent assays of fibroblast conditioned media (III, IV)

TGF-β1, activin A and bFGF growth factor secretion by the different fibroblast cell types were analyzed with enzyme-linked immunosorbent assay (ELISA). 0.2 $ml/cm^2$ of hESC medium without bFGF was collected daily after overnight incubation on the hFF CRL-2429 and hDF 001/08 feeder cells. The media were collected on days 4–7 post inactivation and stored at −70 °C prior the ELISA analysis (III). RPEbasic media conditioned on hFF and mEF cells as described in Chapter 4.1.3.4 were analyzed (IV).

Commercial ELISA kits were used (all from Quantikine, R&D Systems) according to manufacturer’s instructions. Optical densities were measured using Wallac Victor2™ 1420 Multilabel counter (Perkin Elmer-Wallace, Norton, OH,

USA). All standards and samples were tested in duplicates and measurements repeated twice with similar results. For details, see Articles III and IV.

## 4.4 Analyses of human pluripotent stem cell differentiation to retinal pigment epithelial cells (IV)

### 4.4.1 Pigmentation (IV)

The onset of pigmentation of the RPE cell differentiation cultures was followed daily by microscopic inspections. The pigmented cells could easily be detected from the cell culture dishes and the day of the appearance of the first pigmented cells in each differentiation medium (RPEbasic, mEF-CM and hFF-CM) and experiment was recorded. Also, the percentage of cell aggregates containing pigment in relation to total number of aggregates was counted after 28-31 days of differentiation.

### 4.4.2 PCR analyses (IV)

#### *4.4.2.1 Quantitative PCR (IV)*

Expression of RPE differentiation related genes *RAX*, *PAX6* and *MITF* were studied with q-PCR during at time points 7, 14, and 28 days of differentiation with TaqMan Gene Expression Assays. Expression levels of undifferentiated hPSC samples were used as reference level for each gene. Fold change and fold regulation values were calculated as described above in section 4.2.2. For details, see Article IV.

#### *4.4.2.2 RT-PCR (IV)*

Monolayers of hPSC-RPE cells maturated on human collagen IV were analyzed for expression of RPE specific genes: MITF, OTX2, RPE65, bestrophin, PMEL17, PEDF and tyrosinase by reverse transcription PCR (RT-PCR). GAPDH was used as endogenous control. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, GmbH & Co, Düren, Germany). 40 ng was reverse-transcribed to

cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The synthesis of cDNA was carried out in PCR MasterCycler (Eppendorf AG, Hamburg, Germany): 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and cooled down to 4°C. RT-PCR reaction consisted of 1 µl of cDNA as template, 5 U/µl Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific, Leicestershire, UK) and 5 µM primers (Biomers.net GmbH, Söflinger, Germany). Genomic control reactions excluding the enzyme (-RT) for each RNA sample were performed. Primer sequences and annealing temperatures are presented in Table 9. PCR reactions were performed in PCR MasterCycler ep gradient as follows: 95 °C for 3 min, 38 cycles at 95 °C for 30 s, 30 s annealing and extension at 72 °C for one min, final extension at 72 °C for 5 min. PCR products were analyzed on 2.0% agarose gels with a 50 bp DNA ladder (MassRulerTM DNA Ladder Mix; Fermentas). The bands were visualized with Quantity one 4.5.2. Basic program (Bio-Rad Laboratories, Hercules, USA).

Table 9. RT-PCR primer sequences and annealing temperatures.

| **Gene** | **Forward (5’ > 3’)** | **Reverse (5’ > 3’)** | **Annealing temperature** |
|---|---|---|---|
| *GAPDH* | GTTCGACAGTCAGCCGCATC | GGAATTTGCCATGGGTGGA | 55 |
| *MITF* | AAGTCCTGAGCTTGCCATGT | GGCAGACCT TGGTTTCCAA | 52 |
| *OTX2* | GGGCCCTGGGCTTCTTGTCC | ATTGGCCACTTGTTCCACTC | 52 |
| *RPE65* | TCCCCAATACAACTGCCACT | CACCACCACACTCAGAACTA | 52 |
| *bestrophin* | GAATTTGCAGGTGTCCCTGT | ATCCTCCTCGTCCTCCTG AT | 55 |
| *PMEL* | GTGGTCAGCACCCAGCTTAT | GAGGAGGGGGCTATTCTCAC | 52 |
| *PEDF* | AGCTCGCCAGGTCCACAAAG | TGGGCAATCTTGCAGCTGAG | 60 |
| *tyrosinase* | TGCCAACGATCCTATCTTCC | GACACAGCAAGCTCACAAGC | 52 |

## 4.4.3 Immunofluorescence (IV)

### *4.4.3.1 Quantitative analysis (IV)*

PAX6 and MITF expression was studied by immunofluorescence after 28 days of hESC-RPE cell differentiation in the three media. Cell aggregates were dissociated to single cells and centrifuged onto glass cover slips with Shandon-Cytospin-2 cytocentrifuge (Thermo Fisher Scientific). The cells were immunolabeled and randomly imaged for counting of positive cells to total number of cells. ImageJ Image Processing and Analysis in Java software

(http://imagej.nih.gov/ij/index.html) was used for counting. For details, see Article IV.

#### *4.4.3.2 Evaluation of maturation (IV)*

Monolayers of hPSC-RPE cells maturated on mouse collagen IV cell culture inserts were analyzed with immunofluorescence for the expression and correct localization of RPE-specific proteins: CRALBP, MITF, bestrophin and ZO-1. The cells were fixed with 4% PFA, 10 min at RT. The cells were permeabilized with 0.1% Triton X-100 in DPBS at RT for 10 min and blocked with 3% BSA at RT for 1 h. Samples were incubated for 1 h at RT with primary antibodies: mouse anti-CRALBP (1:1000), rabbit anti-MITF (1:350), rabbit anti-bestrophin (1:500) (all from Abcam) and mouse anti-ZO-1 (1:250) (Life Technologies). 1:1500 diluted secondary antibodies: Alexa Fluor 568-conjugated goat anti-rabbit IgG, and Alexa Fluor 488-conjugated donkey anti-mouse IgG and goat anti-rabbit IgG (all from Molecular probes, Life Technologies) diluted in 0.5% BSA in DPBS were incubated for 1.5 h at RT DPBS was used for washings and Vectashield mounting medium with DAPI for nuclei counterstaining. Images were taken either with Olympus BX60 microscope or LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) using a 63x oil immersion objective.

## 4.5 Statistical analyses (II, III)

Pasw statistics 18 software and Mann–Whitney U-test were used to determine statistical significance for difference in relative gene expression of pluripotency markers in hESCs (II, III), for difference in mean proliferation rates of hESCs (III), and for difference in mean secretion of growth factors by feeder cells (III). For ECM array analysis Student's t-test was used by the RT² Profiler™ PCR Array Data Analysis software to determine statistical significance of the difference in relative expression of ECM genes by hFF CRL-2429 and hDF 001/08 feeder cells (III). P-values <0.05 and <0.01 were considered statistically significant and highly significant, respectively.

## 4.6 Ethical considerations (I-V)

The IBT (formerly Regea) has the approval of the National Authority for Medicolegal Affairs in Finland to do research with human embryos (Dnro 1426/32/300/05), and supportive statements of the Ethics Committee of Pirkanmaa Hospital District for the derivation, culture, characterization, and differentiation of hESC lines (R05051, R05116). The embryos used for deriving hESC lines were surplus embryos donated voluntarily by couples undergoing IVF treatments. The embryos were unsuitable for IVF treatments based on either compromised quality or prolonged storage. Both partners signed an informed consent and no financial compensation was paid.

Pediatric dermal tissues for derivation of fibroblasts were donated with informed consent of voluntary parents of children in surgery at Tampere University Hospital, Tampere, Finland. No financial compensation was paid and the project had a supportive statement of the Ethics Committee of Pirkanmaa Hospital District (R05149). Human iPSCs were derived and characterized at the University of Helsinki, with the permission of the Ethics Committee of the University of Helsinki.

# 5. Results

## 5.1 Human serum suboptimally supports undifferentiated human embryonic stem cell culture (I)

Three xeno-free culture media (X-Vivo-10, X-Vivo-20, TeSR1) and five serum replacements (Lipumin, SerEx, SR3, SSS, Plasmanate®) were tested for their ability to support undifferentiated hESC culture. None of these were able to maintain the hESCs on human feeder cells. The hESCs underwent excessive differentiation already during the adaptation phase, leading to corresponding changes in colony morphology. Accordingly the expression of Nanog was lost and expression of SSEA-1 increased in immunostainings. The growth of the colonies was also reduced and the morphology of the feeder cells affected by some of the test media. All hESC colonies differentiated by the end of the first adaptation passage and could not be cultured further in any of the media except TeSR1. In TeSR1 the hESCs were cultured up to passage level seven, after which all colonies differentiated and the culture was aborted.

Culture media containing 10% and 20% HS were also tested. The hESC colonies underwent excessive differentiation during the first passages but in the medium containing 20% HS, the hESCs regained their undifferentiated morphology after serial passaging. At passage level 11, most of the colonies showed undifferentiated morphology, although they were notably thinner than hESCs cultured in the hESC medium. The colonies also stained partly positive for undifferentiated hESC markers of Nanog, Oct-3/4, SSEA-4 and negative for SSEA-1. Flow cytometry analysis at passage level 11, however, showed that only 35% of the hESCs cultured in 20% HS were positive for SSEA-4 compared to 80% in hESC medium (See data in Article I). The 20% HS culture medium was thus found to sustain undifferentiated hESC proliferation to some extent, but to be inferior to the standard hESC medium containing 20% ko-SR.

## 5.2 Commercial feeder-independent culture methods support undifferentiated human embryonic stem cell culture (II)

Selected biomaterials (Ti, $TiO_2$, $ZrO_2$, PDTEC plate, and PLDLA scaffold) and several previously published hESC culture methods and various modifications of these were tested for feeder-independent culture of hESCs.

The hESCs did not attach to any of the biomaterials in the presence of unconditioned hESC medium, and with the hESC-CM only weak attachment and fragile colony growth was achieved in the first passage, with no attachment after further passaging.

Fibronectin surfaces in combination with modified hESC medium (mhESC) as described by Amit and co-workers (Amit et al., 2004; Amit and Itskovitz-Eldor, 2006) failed to support hESC culture beyond the second passage (8 days). Similarly, on fibronectin combined with chemically defined medium (CDM) reported for hESC culture by Vallier and co-workers (Vallier et al., 2005), hESCs quickly underwent differentiation and attached poorly after first passaging. CDM also failed to support hESC culture in combination with HS or FBS surfaces as tested with karyotypically normal hESC lines. However, both matrices and CDM did support the culture of the karyotypically abnormal HS237 hESC line (46X, abnormal X).

HS coated culture dishes were also tested as described by Stojkovic and co-workers (Stojkovic et al., 2005b), with the modification of using hESC medium or hESC-CM instead of conditioned medium recovered from fibroblast-like cells derived from hESCs. On HS coating hESCs quickly differentiated in both media, with the hESC-CM supporting the hESC culture for a maximum of three passages.

A mix of human ECM components of collagen IV, vitronectin, fibronectin, and laminin was tested in combination with defined, xeno-free TeSR1 medium as described by Ludwig and co-workers (Ludwig et al., 2006a). On hECM mix and TeSR1, hESCs were cultured for a maximum of 7 passages. The hESCs showed poorer attachment and underwent progressive differentiation to fibroblast-like cells at each passage and eventually lost the gene and protein expression of Oct-3/4.

Matrigel™ matrix combined to hESC medium or hESC-CM could not support undifferentiated hESC culture beyond the second and fifth passages, respectively. However, Matrigel™ combined with mTeSR™1 medium, a xeno-compound

containing version of the TeSR1 described by Ludwig and co-workers (Ludwig et al., 2006b) supported long-term, undifferentiated hESC culture. With this culture system, the HS401 hESC line was successfully cultured for 36 passages and Regea06/015 hESC line for 38 passages (both approximately 5 months) with proper attachment and undifferentiated colony morphology. Q-PCR analysis showed constant expression of pluripotency markers Oct-3/4 and Nanog compared to hESC culture on hFF feeder cells. The cells strongly expressed Nanog, Oct-3/4, TRA-1-60, TRA-1-81 and SSEA-4 and did not express SSEA-1 in immunostainings throughout the culture. Flowcytometry analysis confirmed over 90% of cells to be positive for SSEA-4 and TRA-1-81 after 23 culture passages. Both cell lines were found karyotypically normal diploid (46, XY) after long term culture: HS401 after 24 passages and Regea06/015 after 35 passages. The cell lines were successfully cryopreserved in mFreSR freezing medium (STEMCELL Technologies). The characterization of Regea 06/015 hESC line is shown in Figure 5.

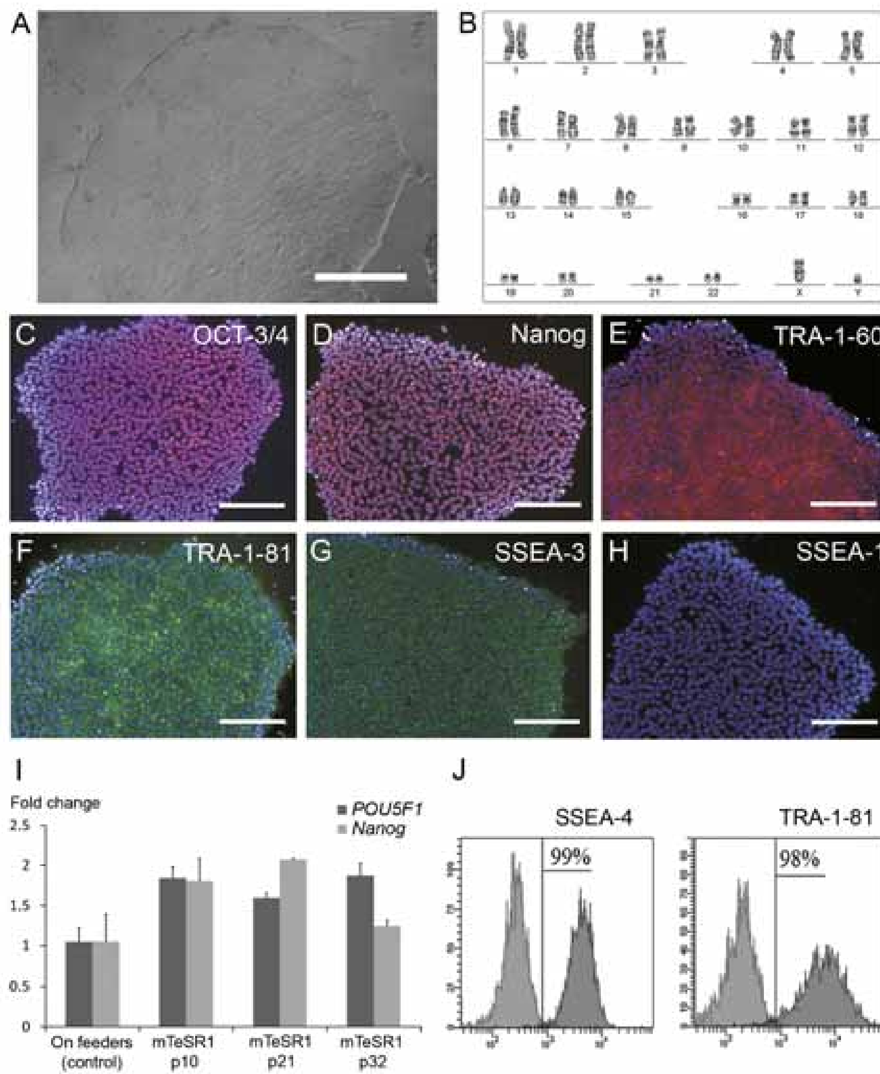


Figure 5. Characterization of Regea06/015 hESC line after long-term culture on Matrigel™ in mTeSR™1 medium. A) A phase contrast image showing typical undifferentiated hESC colony morphology after 38 passages. Scale bar 500 µm. B) Normal karyotype (46, XY) after 35 passages. C-H) Overlay images of immunofluorescence stainings with hESC markers and DAPI nuclei staining after 32 passages. Scale bars 200 µm. I) Relative Q-PCR analysis of *POU5F1*and *Nanog* gene expression during the culture, p = passage. Human ESCs cultured on feeder cells are used as a reference, fold change = 1. J) Flowcytometry analysis for SSEA-4 and TRA-1-81 expression after 23 passages.

Similarly, the other commercial culture system based on CELLstart™ matrix in combination with StemPro® hESC SFM medium (Wang et al., 2007) was able to support long-term hESC culture. Regea 06/015 hESC line was cultured with the culture system for 23 passages (3 months) with undifferentiated colony morphology and marker expression (Figure 6). The morphology of the colonies varied from smooth and even to ragged and uneven, but immunostainings confirmed undifferentiated status despite the varying colony morphology. After 23 culture passages the quality of the culture deteriorated due to a change in the culture medium supplement batch. Karyotyping after 24 passages showed that from 20 analyzed cells, 19 had normal diploid karyotype (46, XY) while one cell had gained a trisomy 18 (47, XY). The cell culture was thus ended at passage level 27.

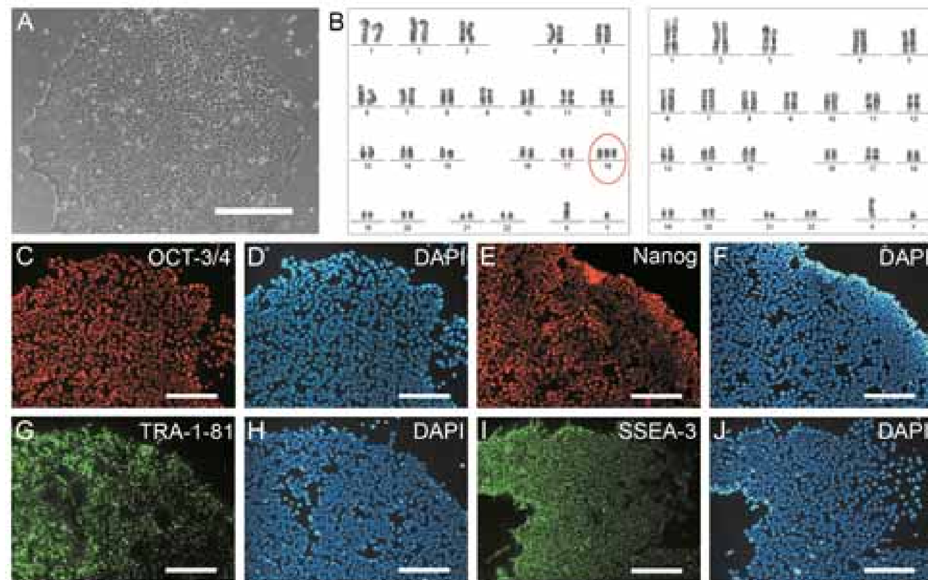


Figure 6. Characterization of Regea06/015 hESC line after culture on CELLstart™ in StemPro® hESC SFM medium. A) A phase contrast image showing typical undifferentiated hESC colony morphology after 15 passages. Scale bar 500 µm. B) Karyotype after 24 passages, one out of 20 metaphases analyzed showed trisomy 18 (red circle), while 19 showed normal karyotype (46, XY). C-J) Immunofluorescence images showing hESC marker expression and corresponding nuclei counterstainings with DAPI after 10 passages. Scale bars 200 µm.

## 5.3 Establishing and optimizing xeno-free and Good Manufacturing Practice-compatible fibroblast feeder cells and their culture methods (III)

### 5.3.1 Derivation of human dermal fibroblasts (III)

To establish xeno-free feeder cells we derived primary fibroblasts from pediatric dermal tissue pieces. A total of 6 pediatric skin tissue biopsies were donated in context of surgery and five hDF cell lines (hDF 001/06, hDF 002/06, hDF 003/06, hDF 001/07, and hDF 001/08) were successfully derived from the tissues. Culture medium was supplemented with HS and animal-origin-free, recombinant enzymes were used for xeno-free culture. The derivation of one cell line, hDF 002/07 failed as enzymatic dissociation techniques and defibrinated, delipized HS were unsuccessfully tested for the derivation. Explant culture proved superior to enzymatic digestion of dermis with collagenase. The outgrowth of fibroblasts was generally detected within a week. Fibroblast growth typically slowed down after 6–7 passages. Low passage stocks of all five hDF lines were successfully cryopreserved.

### 5.3.2 Human embryonic stem cell support of the feeder cells (III)

All five hDF lines were tested for supporting long term, undifferentiated hESC culture with multiple, independently derived hESC lines. Unfortunately none of the hDF lines maintained long term hESC culture. Human ESC colonies underwent differentiation within the first passages leading to typical colony morphology changes and a progressive decrease in pluripotency gene and protein expression (Figure 2 in Article III).

As the in-house derived fibroblasts failed to support hESC culture, commercially available C-12300 hFF cells were tested as an alternative hFF cell line for possible transfer to GMP-quality culture. The C-12300 hFF line was initially chosen for testing as this cell line has been subjected to rigid quality control and pathogen testing by the supplier. The C-12300 hFF cells were initially derived to FBS-medium but thawed upon receipt to both HS-and FBS-containing culture media. The C-12300 hFFs cultured in either FBS-or HS-media supported hESC culture only

suboptimally for less than 10 passages. The feeder cells cultured with HS-medium did, however, support the derivation of a new, xeno-free hESC line Regea 06/015 in combination with the RegES medium (Rajala et al., 2010).

CRL-2429 hFF cells cultured in FBS-medium are routinely used for derivation and culture of hESC lines in our laboratory (Skottman, 2010). Also, as thawed upon receipt to, and cultured with HS-medium, the CRL-2429 hFFs supported undifferentiated culture of several hESC lines. Human ESC colonies were typically thinner on HS-cultured fibroblasts than on FBS-cultured feeder cells. Manual passaging was more challenging but the hESCs showed undifferentiated morphology with corresponding pluripotency gene and protein expression after continuous passaging (Figure 2 in Article III).

### 5.3.3 Human serum containing culture medium (III)

The two commercial hFF cell lines, CRL-2429 and C-12300, and the in-house derived hDF cells were used for testing and optimizing fibroblast culture media. The various HS and concentrations, additives to culture media, and serum-free culture media listed in Table 5 were tested. None of the serum-free media, serum- and xeno-free hESC culture medium RegES with different additives, FibroLife® designed for serum-free fibroblast culture, StemPro® MSC SFM designed for serum-free culture of mesenchymal stem cells, or any of the serum replacements namely 10% ko-SR, 20% Lipumin™, 20% SerEx and 20% SSS, were able to support serial passaging and expansion of the fibroblast cultures. The cells either elongated, showed abnormal morphology and died at the first passage, or did not proliferate after the first or second passage.

HS-containing culture medium did support fibroblast cultures. Compared to the standard FBS culture, fibroblasts cultured with HS proliferated more slowly, underwent replicative senescence earlier (passage 13 vs. passage 15 in 10% FBS), were larger in size, clustered together and detached from the culture vessels more easily. HS concentrations of 10% and 15% were found adequate for optimal growth. Variations in the capacity of sera from different manufacturers as well as batch-to-batch variations for single manufacturer's sera were notable. All sera except charcoal stripped, defibrinated, delipized HS were able to support fibroblast growth.

Heat inactivation (30 min at 56 °C) of HS substantially decreased cell growth rate and impaired the quality of the culture. Filtering the sera or the medium resulted in clearer medium but did not negatively affect cell growth.

The presence of xenogeneic sialic acid neu5Gc was measured from C-12300 and hDF 003/06 cells cultured in HS- and FBS-media and also from hDF cells transferred from HS- to FBS-culture medium. Cells cultured in FBS-medium contained 2-10 ng/1x$10^6$ cells of Neu5Gc. Cells in HS-medium did not contain detectable levels of neu5Gc (<0.2 ng/1x$10^6$ cells). Transfer from FBS- to HS-culture also removed the neu5GC from the cells below the detectable level within two passages (13 days).

The addition of bFGF, TGF-ß1, and activin A growth factors or L-ascorbic acid to 5% HS-medium, were tested in order to decrease the serum concentration, but the additives did not enable optimal fibroblast culture. The growth factors initially accelerated proliferation, but after serial passaging led to altered cell morphology and earlier senescence. Therefore 15% HS-medium was used for subsequent fibroblast culture.

### 5.3.4 Optimizing the feeder cell culture protocols (III)

Besides the fibroblast derivation and medium composition, other aspects of fibroblast culture, cryobanking, and feeder cell preparation were also optimized in order to find simple, cost-effective, xeno-free, and eventually GMP-compatible procedures. Both hFF lines and the in-house derived hDF lines were used for the optimization.

To replace the porcine trypsin routinely used for cell dissociation, a xeno-free recombinant TrypLE Select, which appeared on the market at the time, was successfully used for subculturing. Five percent DMSO supplementation of HS-culture medium was adequate for cryopreservation of fibroblast stocks with slow freezing method. Porcine gelatin traditionally used for coating cell cultureware before fibroblast seeding, was not used since adequate cell attachment was achieved by using CellBIND® cell culture ware.

Gamma irradiation was preferred to Mitomycin C treatment for the inactivation of proliferation due to the consistency of the radiation dose. However, since

irradiation is unsuitable for transfer to clean room and the traditional Mitomycin C treatment (3 hours with 10 µg/ml at 37ºC) was considered too time consuming, different combinations of time and concentration were tested for Mitomycin C treatment: 50 µg/ml for 15-40 min; 100 µg/ml for 10-15 min; 200 µg/ml for 8 min; 400 µg/ml for 4 min and 2.5-40 µg/ml for overnight incubation at 37ºC were tested. After inactivation the cells were split 1:2 and cell proliferation and morphology were monitored for two weeks by daily microscopic inspection and imaging. Mitomycin C treatment with 50 µg/ml for 30-35 min was found optimal for inactivation. Proliferation was inhibited but fibroblast morphology remained good and no cell death was observed. Shorter incubation failed to inhibit proliferation whereas high concentrations over 100 µg/ml or long incubation times (all concentrations used for overnight incubation) caused changes in morphology and considerable cell death.

Freezing of irradiated xeno-free feeder cells as ready-to-use batches to create WCBs was also tested for purposes of clean room culture. This was mostly unsuccessful due to massive cell death during freezing and thawing. Freshly prepared feeder cells were thus used for plating of hESCs. A cell density of $3.65 \times 10^4$ cells/cm$^2$ of inactivated fibroblasts was found optimal. Slow adaptation to serum-free hESC culture medium by gradually decreasing the concentration of HS-medium after irradiation was essential to prevent detachment of the feeder cell layer. The feeder cell layers were used for hESC plating 2-4 days after inactivation.

## 5.4 Human embryonic stem cell supportive and non-supportive xeno-free feeder cells differ in extracellular matrix production and growth factor secretion (III)

To study the factors behind the difference in the hESC supporting capacity of the xeno-free hFF CRL-2429 and hDF 001/08 fibroblasts, differences in key growth factor secretion and ECM production of the two fibroblasts types were studied.

Secretion of hESC-essential growth factors bFGF, TGF-ß1 and activin A was studied by ELISA analysis. Either hFF or hDF cells did not secrete detectable concentrations of bFGF, while both feeder cell types secreted similar concentrations

of activin A. The hFF cells secreted 0.6 ng/ml more TGF-ß1 than hDF cells, but the addition of the same concentration of TGF-ß1 to hESC culture on hDF cells did not rescue hESC colony morphology or pluripotency marker expression. Partial rescue of colony morphology and expression of TRA-1-81 required supplementation six times a week with tenfold the concentration (6.0 ng/ml) of recombinant TGF-ß1 (Figure 5 in Article III). We thus concluded that it was unlikely that the difference between the feeder cells was attributable to the difference in secretion of the growth factors tested.

The hFF and hDF feeder cells were compared for expression of altogether 88 ECM and ECM-related protein-coding genes by q-PCR array analysis and q-PCR assays of selected laminin subunit coding genes. Laminin subunits α1, α3, α5, ß1, ß3 and laminin binding integrin subunits α3, α6, α7 were found to be down-regulated at least two fold in the hDFs compared to the hFF cells (Figure 3 in Article III). These results indicate lower expression of laminins −111, −332 and −511 and laminin binding integrins α3ß1, α6ß1, α6ß4, α7ß1 in the hDF cells. The production of the laminin chain α5 (laminin-511) by the feeder cells was further studied at protein level. Immunoprecipitation from culture media showed and immunofluorescence labeling verified that hESC supportive hFF and mEF fibroblasts synthesize laminin α5, whereas the four hDF lines analyzed did not (Figure 4 in Article III). In addition to the laminin production, other genes of interest were down-regulated in the hDF cells compared to hFF. For example E-cadherin type I, vitronectin and Kallmann syndrome 1 sequence (KAL1) coding for FGF regulator Anosmin-1, were down-regulated 2.5, 2.7 and 26-fold, respectively in the hDF cells.

## 5.5 Mouse and human feeder cells enhance human pluripotent stem cell differentiation to retinal pigment epithelial cells (IV)

The effect of the commonly used mouse and human feeder cells on the differentiation of hESCs and hiPSCs to RPE cells was studied by comparing suspension differentiation in three media: RPEbasic (15% ko-SR, no bFGF), mEF-CM and hFF-CM.

The onset of pigmentation was faster in the conditioned media (CM) than in RPEbasic for both hESCs and iPSCs, with the first pigments appearing around two weeks of differentiation. Increase in relative expression of early eye field markers PAX6 and RAX analyzed with q-PCR confirmed that the differentiation progressed to eye field direction in all media. By day 28 of differentiation the RAX expression had decreased in both CM accompanied by a 10-fold increase in the expression of RPE specific MITF, with strongest expression in the mEF-CM (Figure 2 in Article IV). After four weeks of differentiation, the CM consistently contained larger pigmented areas and a greater number of pigmented cell aggregates. The ratios of PAX6 and MITF positive cells were also clearly higher in the CM when studied by counting positive cells after immunofluorescence staining (Figure 3 in Article IV).

As the factors secreted by the fibroblasts enhanced the early hPSC-RPE cell differentiation, the media were analyzed for bFGF, TGF-ß1, and activin A growth factor secretion with ELISA analysis. The mEF cells were found to secrete approximately 6 ng/ml more activin A than hFF cells. Activin A, hypothesized as RPE inductive agent in the mEF-CM, was tested for induction of RPE cell differentiation by supplementing RPEbasic with 10 ng/ml of recombinant human activin A. The activin A supplementation accelerated the onset and degree of pigmentation. At the 28-day time point, cultures treated with activin A contained 96% (±1%) PAX6 and 71% (±14%) MITF positive cells, while control cultures in RPEbasic contained 74% (±16%) PAX6 and 57% (±13%) MITF positive cells (Figure 5 in Article IV).

After selective plating of pigmented areas to adherent cultures, the cells morphologically underwent an epithelial-mesenchymal transition (EMT) to fibroblast-like morphology, and after proliferation to confluence, regained the cobblestone morphology and pigmentation typical of RPE cells. The matured hPSC-RPE cells showed gene expression of RPE-specific marker genes: MITF, OTX2, RPE65, bestrophin, PMEL17, PEDF and tyrosinase when analyzed with RT-PCR. Immunofluorescence analysis showed correct expression and localization of MITF in the nuclei; bestrophin and CRALBP both in the cytoplasm and cell membranes; and tight junction protein ZO-1 in the cell junctions (Figure 4 in Article IV).

# 6. Discussion

## 6.1 Human serum as xeno-free culture media supplement for human embryonic stem cells (I)

None of the eight commercially available hESC culture media or serum replacements available at the time of testing, was able to maintain long-term, undifferentiated hESC culture on human feeder cells. Although all these formulations were designed as serum substitutes for cell culture, with the exception of TeSR1, none were specially designed for the culture of hESCs. The X-Vivo™ 10 medium had been previously used for hESC culture (Genbacev et al., 2005; Li Y et al., 2005; Fletcher et al., 2006; Peerani et al., 2007) but in feeder-independent culture systems and combined with high concentrations of additional growth factors. The X-Vivo™ 10 medium has a defined composition containing only human sourced and recombinant proteins including albumin, recombinant insulin, and transferrin. Plasmanate® was used by Klimanskaya and co-workers in combination with ko-SR, FGF and LIF for the first feeder-independent derivation of six hESC lines (Klimanskaya et al., 2005). Similarly, the TeSR1 is also designed for feeder-independent hESC culture. Unfortunately the chemical compositions of most media and supplements are proprietary, making it impossible to evaluate which essential compounds are missing to enable hESC self-renewal and pluripotency.

We found culture medium supplemented with 20% HS to suboptimally support undifferentiated hESC culture up to passage level 11. HS has previously been used in hESC culture with some success. Richards and co-workers derived a hESC line using 20% HS-containing culture medium and were able to propagate hESCs in undifferentiated state for ten passages (Richards et al., 2002). It was later observed that prolonged use of HS beyond the tenth passage led to increased differentiation of hESCs (Richards et al., 2003). Other studies have likewise found the use of HS to increase spontaneous differentiation in hESC cultures (Koivisto et al., 2004; Chen HF et al., 2009). Koivisto and co-workers cultured hESCs for 4 passages in 20%

HS-supplemented medium with only 38% of colonies showing undifferentiated morphology and being notably thinner compared to colonies in 20% ko-SR (82% undifferentiated colonies) (Koivisto et al., 2004). This is consistent with our flow cytometry data of 35% versus 80% of SSEA-4 positive hESCs in 20% HS and ko-SR media respectively. We supplemented the culture medium with the antioxidant L-ascorbic acid, which is also included in ko-SR (Price et al., 1998). The culture of Koivisto and co-workers was instead supplemented with ITS, which had a negative effect on the growth of the colonies (Koivisto et al., 2004). Ellerström and co-workers were able to derive and propagate a new hESC line for over 30 passages in 20% HS-containing culture medium without problems of excessive differentiation. They also used HS for the derivation and culture of their primary hFF cells, thereby deriving the first truly xeno-free hESC line (Ellerstrom et al., 2006). They used custom made, superior quality HS (Tallheden et al., 2005).

Although HS is a xeno-free alternative to FBS, it contains a complex mixture of hormones, growth factors, differentiation and attachment factors, as well as undefined components. The quantity of albumin, which is the major blood protein, seems to be essential for hESC survival *in vitro*. This notion is supported by the fact that BSA is the major constituent of ko-SR (Price et al., 1998). In our study the HS concentration of 20% contained a sufficient quantity of serum proteins with a tolerable quantity of differentiating factors.

The optimal solution would be to replace the ko-SR in hESC culture with a similar, defined serum replacement containing only purified human or human recombinant components. Such xeno-free hESC media alternatives have since been developed for both feeder-dependent and feeder-independent culture and are also commercially available. The RegES medium (Rajala et al., 2010), HEScGRO™ medium available from Millipore, and KnockOut™ SR XenoFree CTS™ available from Life Technologies were developed for hESC and iPSC culture with either hFF or mEF feeder cells. Reports on successful use of these media for long-term culture of both hESCs and hiPSCs have been published (Chin et al., 2010; Rajala et al., 2010; Rodriguez-Piza et al., 2010; Kibschull et al., 2011). Media developed for feeder-independent hESC culture are discussed next in Chapter 6.2.

## 6.2 Feeder-independent human embryonic stem cell culture (II)

The only feeder-independent hESC culture methods found to support long-term undifferentiated hESC culture were the two commercial methods available at the time of the study: Matrigel™ combined with mTeSR™1 and CELLstart™ combined with StemPro® hESC SFM, the latter being at a testing phase. Both media contained xeno-compounds, but CELLstart™ is a human sourced matrix. All the other culture methods and their modifications as well as the biomaterials (Ti, $TiO_2$, $ZrO_2$, PDTEC and PLDLA) tested in combination with hESC-CM failed to support proper hESC attachment and culture.

Clearly the biomaterials lacked the functional ECM ligands necessary for hESC attachment and growth as the ECM substrates resulted in better attachment of hESCs. Conditioning of the hESC medium on hFF cells did not provide the cells with the necessary nutrient and signal molecules as the hESC-CM failed to support hESC culture even on Matrigel™. Similarly the fibronectin and sera substrates used for the other non-hESC supporting methods clearly provided insufficient attachment factors, and the media used lacked nutrients and growth factors. The mhESC medium and CDM are based on very low concentrations of TGF-ß and bFGF or activin A respectively to maintain pluripotency.

The fact that HS and FBS surfaces and the CDM did support the culture of the karyotypically abnormal HS237 (46X, abnormal X) hESC line, indicated that the karyotypic abnormality enabled culture adaptation. It is known that hESCs commonly undergo adaptive changes during prolonged culture *in vitro* and show increased growth rate, reduced apoptosis and karyotypic changes (Draper et al., 2004b; Baker et al., 2007; Catalina et al., 2008; Narva et al., 2010; International Stem Cell Initiative et al., 2011). Also, the harsh feeder-independent culture conditions have been shown to favor the occurrence of karyotypic abnormalities (Draper et al., 2004b; Ludwig et al., 2006a; Catalina et al., 2008). In our study, the hESC line cultured on CELLstart™ in StemPro® hESC SFM medium had gained a trisomy 18 (47, XY) to one out of 20 metaphases studied by passage level 24.

A large multi-laboratory comparison conducted by the International Stem Cell Initiative (ISCI) Consortium (International Stem Cell Initiative Consortium et al., 2010) evaluating diverse feeder-independent hESC culture methods obtained results

that concur with ours. In that study, eight culture systems (Li Y et al., 2005; Vallier et al., 2005; Liu et al., 2006; Lu et al., 2006; Ludwig et al., 2006a; Ludwig et al., 2006b; Yao et al., 2006; Wang et al., 2007) (See Table 2) were tested with ten hESC lines. Only the two commercial media, mTeSR™1 and StemPro® hESC SFM, supported feeder-independent hESC maintenance for ten passages. The method published by Vallier and co-workers based on fibronectin/FBS matrix in combination with CDM was found to be insufficient for long-term hESC culture as were the other methods published by various academic research groups (International Stem Cell Initiative Consortium et al., 2010). In accordance with our results, other studies have since evaluated hESC culture methods and have found the mTeSR™1 and StemPro® hESC SFM culture methods simple and functional for long-term hESC culture (Chin et al., 2010; Hannoun et al., 2010; Hernandez et al., 2011).

The commercial media have gone through considerable development, manufacture, and quality control procedures. Similar reproducibility is difficult to achieve in an academic laboratory often with limited resources and only a few cell lines (International Stem Cell Initiative Consortium et al., 2010). When tested at our laboratory, the CELLstart™ and StemPro® hESC SFM culture system was at a developmental state and has since been further developed and improved. It is thus likely that the batch change of an incompletely optimized, critical medium supplement caused the sudden crash of the cell culture in our study at passage level 23.

Both mTeSR™1 and StemPro® hESC SFM media contain substitutes for serum components like transferrin, cholesterol, and lipids, and, importantly, bovine serum albumin. The media contain important growth factors to stimulate the FGF pathway via bFGF, and TGF-ß pathway via TGF-ß or activin A. The two media also activate alternate signaling pathways as mTeSR™1 uses a GABA agonist and the non-specific Wnt antagonist lithium chloride, while StemPro® hESC SFM uses the ERBB2/ERBB3 ligand heregulin-1β and the IGF ligand IGF1 LR3 (Ludwig et al., 2006b; Wang et al., 2007; International Stem Cell Initiative Consortium et al., 2010). The commercial matrices Matrigel™ and CELLstart™ also provide a rich ECM substartes for the hESCs. Matrigel™ has been used with a wide variety of hESC culture media and is an extremely complex substrate containing over 1.800 proteins that vary from batch-to-batch (Hughes et al., 2010). CELLstart™ is a

cGMP-produced, xeno-free and defined matrix containing fibronectin and albumin as the most abundant proteins (Hughes et al., 2011). Both the mTeSR™1 and StemPro® hESC SFM have since been shown to support hPSC culture on human derived and recombinant ECMs as well as engineered and synthetic substrates. mTeSR™1 with fibronectin (Hughes et al., 2011), human recombinant vitronectin (Braam et al., 2008), engineered E-cadherin (Nagaoka et al., 2010), and synthetic phage displayed peptide sequences (Derda et al., 2010). StemPro® hESC SFM with recombinantly generated domain of vitronectin (Prowse et al., 2010) and synthetic polymer coating of poly[2-(methacryloyloxy)ethyl-dimethyl-(3-sulfopropyl)ammoniumhydroxide] (PMEDSAH) (Nandivada et al., 2011). Such synthetic biomaterials, potentially functionalized with ECM functional domains, represent the next generation of substrates for hPSC culture offering reproducibility and more cost-effective surfaces over human-sourced protein matrices.

The xeno-free version of the TeSR1 medium in combination with the hECM mix failed to support hESC culture in our study. Reproducibility factors in the preparation of the medium may have influenced the results, but it is a fact that completely defined, xeno-free, and feeder-independent hPSC culture systems have been difficult to develop and have entered the market only very recently. A variant of the xeno-free TeSR1 medium has enabled long-term culture of both hESCs and hiPSCs on a defined substarate of laminin-511 (α5ß1γ1) (Rodin et al., 2010). Currently there is a xeno-free version called TeSR™2 of the medium and a defined matrix StemAdhere™, consisting of a single recombinant human protein, available from Stem Cell Technologies. TeSR™2 has been successfully used for xeno-free hESC maintenance combined with ECM from hESC-df (Fu et al., 2011).

Similarly, the xeno-free version of the traditional ko-SR (KnockOut™ SR XenoFree) is marketed by Life Technologies in combination with CELLstart™. NutriStem™ XF/FF Culture Medium provided by Stemgent (Cambridge, MA, USA) is a fully defined, xeno-free culture medium for feeder-independent culture of hPSCs used in combination with Matrigel™. ScienCell™ Research Laboratories (Carlsbad, CA, USA) offers a serum-free (but not xeno-free) hPSC medium called STEMium™ in combination with Matrigel™. Further, the very recent development of the E8 medium based on the initial recipe of mTeSR™1, is a huge leap forward as the need for undefined BSA is eliminated. E8 consists of 8 essential components of insulin, selenium, transferrin, l-ascorbic acid, FGF2 and TGF-β (or nodal) in

DMEM/F12 and enables long-term hESC and hiPSC culture as well as feeder-independent derivation of hiPSCs (Chen et al., 2011).

In spite of major improvements in the development of defined culture systems, successful feeder-independent derivation of hESC lines is still rarely reported (Klimanskaya et al., 2005; Ludwig et al., 2006a; Lagarkova et al., 2010) and efficient derivation requires the use of supporting feeder cells. Moreover, there are doubts about the karyotypic stability of the cell lines derived without feeder cells (Ludwig et al., 2006a) and no GMP-grade feeder-independent and xeno-free hESC lines have been established. The feeder-independent derivation of hiPSCs has been reported frequently in recent years (Sun et al., 2009; Hayashi et al., 2010; Sugii et al., 2010; Beltrao-Braga et al., 2011; Chen et al., 2011; Chung et al., 2012; Macarthur et al., 2012), but other concerns related to the reprogramming still make hiPSCs unsuitable for clinical use. It remains to be seen whether some of these xeno-free, defined media and matrix combinations will stand out and prove to be globally reproducible with different cell lines to enable large scale derivation and culture of stable clinical quality hPSC lines.

## 6.3 Xeno-free feeder cells and their culture conditions (III)

### 6.3.1 Xeno-free feeder cell lines (III)

We established xeno-free hDF cell lines from dermal biopsies. Skin as a tissue source is easily accessible and establishing fibroblasts is technically simple and effective. Thus feeder cells derived from human foreskin tissue have become the most commonly used human feeder cell type for hESC derivation and culture (Hovatta et al., 2003; Inzunza et al., 2005; Crook et al., 2007; Aguilar-Gallardo et al., 2010; Strom et al., 2010) and were recently produced and validated as the first GMP-grade feeder cells (Prathalingam et al., 2012). Foreskin however, is a scarcely available tissue source in Finland, as infant circumcisions are rare. Dermal tissue was chosen as hDF feeder cells have been used for hESC propagation by other research groups (Richards et al., 2003; Tecirlioglu et al., 2010). The procedure of tissue harvest in the operating theater is optimal considering GMP and also the

transfer in a closed vial to the laboratory is easy. Patients with low risk medical history and no infections or malignancies can be chosen.

Although five hDF lines were successfully established, the fibroblast yield depended on the size of the tissue biopsy and varied considerably. Calculations of the cell yield in relation to tissue weight would have been desirable but were not performed. Also, the proliferation rate was lower compared to standard hFF (CRL-2429) but was not quantified. The hDF cells underwent replicative senescence early and were unable to support undifferentiated hESC culture.

We therefore adopted an approach to test commercial hFF lines in order to transfer these to xeno-free culture conditions. Such upgrading of a previously FBS-primed cell line to GMP-compliance is possible but a completely xeno-free feeder cell line cannot be established and excessive testing for xeno-pathogens would be necessary. The other commercial hFF line C-12300 was only suboptimal in hESC support. The C-12300 hFFs supported the derivation of a new xeno-free hESC line, which at passage level 2 was transferred to CRL-2429 feeders, but did not support long-term hESC culture in our study. It is possible that other external factors have affected the fragile hESC culture systems on these feeder cells and led to hESC colony differentiation after a few passages. Commercial CRL-2429 hFF feeder cells have been used for the derivation and propagation of 9 new Regea hESC lines in our laboratory (Rajala et al., 2010; Skottman, 2010) and supported long-term hESC culture, also after transfer to xeno-free culture system based on the use of HS-culture medium.

It is known from earlier studies that there are notable differences between hFF lines to support hESC culture (Eiselleova et al., 2008). The reasons for this are not completely understood but evidently originate from differences in the secretion of growth factors and ECM proteins by the feeder cell lines. The choice of a tissue source with high capacity to support hESCs is essential, but as our results demonstrated, every cell line still needs to be individually tested.

### 6.3.2 Human serum for fibroblast culture (III)

A chemically defined, standardized, xenogeneic-, and serum-free media composition would be the preferred solution for the clinical scale propagation of

fibroblast feeder cells. In this study none of the serum-free media available at the time of testing enabled sufficient culture of feeder cells in quantities necessary for production of feeder cell WCBs and MCBs. It is likely that the nutritional requirements of the strongly proliferating fibroblast cells were not met in these serum-free media. FibroLife® medium was the only medium specifically designed for the serum-free culture of fibroblasts, but even in this medium proliferation halted after two passages. Recently xeno- and serum-free media such as modification of the E8 medium or StemPro® MSC SFM Xeno-Free have been used for hiPSC generation (Chen et al., 2011; Macarthur et al., 2012). Human iPSC reprogramming requires significantly less fibroblasts whereas the establishment of feeder cell MCBs requires cells to be created at $10^9$ magnitude (Prathalingam et al., 2012). To date there are no reports of the use of serum- and xeno-free media for fibroblast feeder cell establishment or culture. Recently the KnockOut™ SR XenoFree was tested for establishing GMP-grade hFF lines but found unsuccessful due to insufficient cell attachment and slow proliferation (Prathalingam et al., 2012).

We used HS supplemented culture medium for xeno-free feeder cell culture. Similarly to our approach, several other research groups have used HS (Richards et al., 2003; Kibschull et al., 2011; Ellerstrom et al., 2006; Meng et al., 2008; Unger et al., 2008a). GMP-grade HS was also used for the very recent derivation and culture of the first reported xeno-free, GMP-grade feeder cells (Tannenbaum et al., 2012). In addition, HS has been used for fibroblast derivation and culture in the context of hiPSC generation (Rodriguez-Piza et al., 2010; Ross et al., 2010). Currently GMP-quality HS, as well as other GMP-quality cell culture reagents and materials, are available from several manufacturers on request.

Fibroblast culture with HS compared to FBS is technically more challenging as morphology, attachment, and proliferation properties are altered and hESC colonies are thinner on HS cultured fibroblasts. Human FFs derived under cGMP using cGMP-manufactured FBS have been approved for clinical use by the FDA (Crook et al., 2007; Prathalingam et al., 2012). It is thus debatable if such qualified bovine-sourced materials guarantee a more robust and safe production of clinical grade feeder and hESC lines. Especially since, in accordance with our study, other studies have shown that the incorporation of xeno-protein Neu5Gc to cells is reversible by subsequent growth under animal component-free conditions (Heiskanen et al., 2007; Prathalingam et al., 2012).

We also optimized other aspects of feeder cell culture, such as cell inactivation and cryostorage methods, with emphasis on the possibility to transfer the procedures to meet GMP-regulations. Yet, many aspects of the feeder cell production process were not considered in the scope of this study. For example, the characterization of the cells including growth kinetics, correct fibroblast marker expression, karyotypic stability, non-tumorigenicity and safety testing issues were not considered.

## 6.4 Growth factor and extracellular matrix production of human embryonic stem cell supporting feeder cells (III)

The hESC supportive hFF and non-supportive hDF feeder cells did not differ in their secretion of bFGF or activin A. Furthermore, the difference in TGF-ß1 secretion was shown not to contribute to differences in hESC support. Instead, differences in the secretion of other growth factors or molecules with known or still unknown function in hESC self-renewal that were not tested for may account for the differences. Interesting candidates are, for example, the IGF family proteins not tested in this study. It should also be noted that the ELISA analyses were conducted in the absence of exogenous bFGF, normally added to the culture media in feeder-dependent culture systems. It has been proposed that exogenously added bFGF functions through the feeder cells. Exogenous bFGF induces the production of other supportive factors like IGF-II and TGF-ß family members including up-regulation of activin A, TGF-ß and Gremlin as well as down-regulation of Bmp4 by the feeder cells or, in the absence of feeder cells, by the autologous feeder cells around the pluripotent hESC colony centres (Bendall et al., 2007; Greber et al., 2007). Autocrine FGF-2 signaling has also been shown to be operative in hESCs (Dvorak et al., 2005). Exogenous and autocrine FGF signaling is upstream of key TGF-ß ligands in hESCs which, in a concerted manner, sustain Oct-3/4, Nanog and Sox-2 expression, whereas these, in turn, activate endogenous expression of bFGF (Greber et al., 2007). Analyzing the growth factor secretion also in the presence of bFGF would have yielded more accurate growth factor secretion profiles. The feeder cells also secrete other molecules and ECM proteins that modulate growth factor signaling e.g. HSPGs (Levenstein et al., 2008; Abraham et al., 2010).

We found differences in the production of distinct laminin isoforms and laminin binding integrin subunits between the feeder cells. Laminin-511 was verified to be produced by the hESC-supportive feeder cells but not by the non-supportive hDF feeder cell lines. Research has shown that undifferentiated hESCs synthesize and deposit laminin-511 and -111, and that purified laminin-511 and even -322 can be used as a supportive matrix in feeder-independent hESC culture (Miyazaki et al., 2008; Evseenko et al., 2009; Vuoristo et al., 2009; Rodin et al., 2010). Accordingly hESCs synthesize laminin binding integrin subunits α3, α6, α7, and interaction with the hESC-critical laminins is mediated through integrins α6ß1 and α3ß1 and Lutheran/B-CAM receptor, an Ig-superfamily member (Xu et al., 2001; Miyazaki et al., 2008; Evseenko et al., 2009; Vuoristo et al., 2009; Rodin et al., 2010). These results indicate that laminin-511 has a central role in the maintenance of hESC survival and pluripotency. Our findings indicate a function also through the feeder cells in feeder-dependent culture systems, yet the possible mechanism remains to be studied. It has been proposed that the role of laminin-511 in feeder-independent cultures is to provide hESCs with focal adhesion contacts to the surface and enable mobility (Rodin et al., 2010)

In addition other genes of interest like those coding for vitronectin, E-cadherin type I and Anosmin-1 showed lower expression in the non-hESC-supportive hDF feeder cells. Purified vitronectin as well as a recombinant, N-terminal domain of vitronectin have been used as defined matrices in feeder-independent hESC cultures (Braam et al., 2008; Prowse et al., 2010). Vitronectin binds to IGF receptors (Kricker et al., 2003) that promote hESC survival and pluripotency (Bendall et al., 2007; Wang et al., 2007). A recent study demonstrated the Vitronectin-IGF-I function in hESC cultures as synthetic vitronectin/IGF-I protein supported long-term hESC culture in the absence of feeder cells and serum (Manton et al., 2010). Testing the hDF and hFF feeder cells for the IGF secretion would thus be interesting as the vitronectin and IGF might co-operate in supporting hESCs.

E-cadherin-mediated cell–cell adhesion and signaling are essential for the colony formation and self-renewal of hESCs (Li L et al., 2012) and a recombinant E-cadherin substratum has been used for the feeder-independent culture of hESCs and hiPSCs (Nagaoka et al., 2010). Moreover, it has been postulated that E-cadherin-based cell contacts are stabilized by laminin-binding integrins possibly binding these two factors together in hESC signaling (Stipp, 2010).

Surprisingly, KAL1 gene coding for Anosmin-1, was down-regulated 26-fold in hDF cells. Anosmin-1 enhances FGF signaling via the regulation of bFGF/FGF receptor-1 (FGFR1)/heparin signaling-complex assembly and activity (Hu et al., 2009). Anosmin-1 could thus play a role in maintaining a sufficient level of bFGF signaling in the hESCs.

These and other ECM proteins/ligands and their synergistic effects offer interesting subjects for future research. Knowledge of the feeder cell function in the co-culture system can facilitate the development of new derivation, culture, and differentiation methods as discussed for feeder aided RPE cell differentiation in the next Chapter 6.5.

## 6.5 Feeder cell induction of human pluripotent stem cell differentiation to retinal pigment epithelial cells (IV)

Human PSCs are often differentiated to RPE cells by allowing overgrowth of the hPSC colonies in co-culture with feeder cells, usually mEFs (Klimanskaya et al., 2004; Gong et al., 2008; Vugler et al., 2008; Buchholz et al., 2009; Liao et al., 2010). It has been reported that the EB method of hPSC-RPE cell differentiation is not as efficient as the adherent culture on mEF feeder cells (Vugler et al., 2008), most likely deriving from the inductive factors provided by the feeder cells. We tested whether the secreted factors of the two most commonly used fibroblast feeder cell types, mEFs and hFFs, had an inductive effect on the hPSC-RPE differentiation. We hypothesized that the fibroblast feeder cells might provide variable mesechymal signals mimicking the extraocular mesenchyme surrounding the RPE and regulating its differentiation during embryonic development (Fuhrmann et al., 2000).

In the presence of fibroblast CM, the RPE differentiation was enhanced compared to differentiation in non-conditioned RPEbasic medium. The mEFs were found to secrete 6 ng/ml more activin A than hFF cells and the addition of 10 ng/ml of recombinant activin A to RPEbasic was thus tested for the induction of RPE cell differentiation. The addition of activin A at this low level had a pronounced, enhancing effect on the early-stage RPE cell differentiation.

The extraocular mesenchyme secretes TGF-ß superfamily growth factors such as activin A, activates the expression of MITF and down-regulates CHX10 expression

directing RPE cell fate differentiation *in vivo*. Similar effects of activin A inducing MITF expression have been reported (Fuhrmann et al., 2000). Activin A has also been shown to induce hPSC-RPE cell differentiation *in vitro*, after initial neural differentiation with nicotinamide (Idelson et al., 2009; Kokkinaki et al., 2011), or after initial differentiation to optic vesicle-like structures with neural media (Meyer et al., 2011). In these previously published studies, high activin A concentrations of 140 ng/ml between day 14 and day 28 of differentiation (Idelson et al., 2009) and 100 ng/ml between day 20 and day 40 (Meyer et al., 2011) were used. However, we were able to induce early hESC-RPE cell differentiation with substantially lower activin A concentration. The superior secretion of activin A by mEF feeder cells could thus be one of the key factors enhancing early RPE cell differentiation and MITF expression while down-regulating CHX10 expression.

We also detected that the hFF cells secreted 342 pg/ml more TGF-ß1 than mEF cells. Active TGF-ß is present in human plasma at concentrations lower than 300 pg/ml (Fortunel et al., 2000). TGF-ß at low physiological concentrations of 300 pg/ml has been shown to have a similar effect on the self-renewal of hESCs in defined medium as high concentration (30 ng/ml) of activin A (Peiffer et al., 2008). Idelson and co-workers also achieved a similar inductive effect on RPE cell differentiation with 2.5 ng/ml addition of TGF-ß1 as with 140 ng/ml addition of activin A, after pre-treatment with nicotinamide (Idelson et al., 2009). It is possible that the TGF-ß1 secreted by the hFF was the inducing growth factor in hFF-CM. The effect of TGF-ß1 was not tested for.

However, in addition to activin A and TGF-ß1 both mEF-CM and hFF-CM may contain a pool of other possible factors inducing RPE cell differentiation. Mouse EFs, for example, secrete PEDF (Lim and Bodnar, 2002; Prowse et al., 2007). Both fibroblast types also secrete various ECM components like collagens I and IV, nidogen I, and fibronectin as well as proteins involved in TGF-ß, BMP, Wnt and IGF signaling (Prowse et al., 2007), known to have important roles in eye specification. Interestingly, IGF signaling in *Xenopus* embryos is involved in eye induction (Pera et al., 2001) and the addition of IGF-1 to the EB cultures has been shown to specifically and efficiently direct hESCs to retinal progenitor identity (Lamba et al., 2006). Thus the field is open for the identification of other important regulators of differentiation.

## 6.6 Future perspectives

The ultimate goal of hPSC research lies in the use of the cells for regenerative medicine to treat devastating diseases that cannot be cured with the methods of conventional medicine. Since their derivation, hESCs have been studied extensively and although there remain concerns regarding their clinical use, the translation of hESC therapeutics to clinical practice has been initiated. Whereas the hiPSC technology has opened up new opportunities for personalized medicine, the therapeutic use of hiPSCs is impeded by additional concern related to the safety of reprogramming. (Drews et al., 2012)

Developing clinical grade hPSC culture conditions to avoid the use of xenogeneic, undefined materials has been a major focus of recent research. Many xeno-free, defined and lately even albumin free culture media have been developed for both feeder-dependent and -independent hPSC culture and have been produced according to GMP-standards. Similarly, more defined synthetic and engineered matrices have been introduced.

Ideally clinical grade hESC lines would be establihed and cultured on a synthetic matrix in combination with a defined, xeno-free culture medium. An ideal culture substratum should be affordable to produce according to GMP, easy to handle and functional in different forms of hESC culture e.g. standard 2D culture on surfaces, versus large scale 3D sphere culture in bioreactors. The material should be easily sterilized, biocompatible, enable easy and complete detachment of hESCs or differentiation and possibly even transplantation of the differentiated hESC progeny to the patient. Similarly, the culture medium should contain only the minimal essential components necessary for the efficient derivation and propagation of karyotypically normal hESC lines and ideally allow for differentiation with modification of growth factor or small molecule cues essential for each application.

Although the feeder-independent culture methods will likely replace the use of feeder cells in the future, the clinically compatible processes established for feeder-dependent hESC culture will not go to waste. The same processes to produce clinical grade human fibroblasts derived now as feeders could later provide important start-up material for new hiPSC lines when their safety issues have been resolved and hiPSC technology can be translated to clinical practice (Unger et al.,

2008b). Studying feeder cell co-culture systems can also provide valuable cues about the molecular mechanism of pluripotency maintenance or induction of differentiation and facilitate the development of efficient, xeno-free and GMP-compatible differentiation strategies. On the other hand, defined conditions allow for reproducible research results and clinical cell line generation with minimal variability in function.

Although the use of hPSCs for cell replacement therapies raises great excitement, there are still many challenges to overcome; scaling-up culture and differentiation protocols, safety concerns about tumorigenicity, genetic and epigenetic stability, recipient immune rejection, issues related to the safety of reprogramming and the high cost of adapting to strict regulatory requirements. International collaboration is required for the development of the regulatory framework to ensure the production of safe, consistent and effective stem cell products but avoiding building regulatory barriers that impede the clinical translation of the hPSC-therapies (von Tigerstrom, 2008).

The need to expand our knowledge on hPSC differentiation to retinal cells is especially topical as the first clinical hESC-RPE trials for retinal regeneration are currently ongoing. In addition to the RPE, also the photoreceptors are impaired in the severe cases of AMD and other retinal degenerative diseases. Consequently more standardized differentiation strategies need to be developed for both RPE and neural retina generation. The potency of hESCs to form organized optic structures and stratified neural retinas, is extremely inspiring (Nakano et al., 2012). Future research will surely focus on creating 3D constructs of hPSC-derived retinas with photoreceptor progenitor cells on the RPE layer. The current phase one clinical trials with hESC-RPE cells will lead the way to additional applications and provide answers to many of the open questions related to the safety and efficacy of hPSC therapies.

# 7. Conclusions

The aim of this study was to test, develop and optimize xeno-free and eventually GMP-compatible culture methods for undifferentiated hESCs and to evaluate the effect of the feeder cells in differentiation of hPSCs to RPE cells. Based on the results of these studies, following conclusions can be drawn:

1. None of the tested xeno-free culture media or serum replacers maintained undifferentiated hESC culture on human feeder cells. HS supplemented culture medium was found to suboptimally sustain undifferentiated hESC growth (I).

2. Two commercial, xeno-compound containing feeder-independent hESC culture systems supported long-term, undifferentiated hESC culture. Other feeder-independent culture methods tested failed to maintain undifferentiated morphology of karyotypically normal hESC lines. Considerable development, manufacture, and quality control procedures conducted on the commercial media and great number of growth factors at high concentrations included, are likely responsible for their capacity to reproducibly support hESC culture (II).

3. Several aspects of xeno-free and GMP-compatible feeder cell production processes were tested and optimized:

    - Xeno-free hDF feeder cells were established using GMP-compatible protocols. However, the feeder cells were unable to support undifferentiated hESC culture beyond early passages, where as hFF feeder cells maintained undifferentiated hESC culture in xeno-free culture conditions. (III)

- HS-supplemented culture medium was found to be the only xeno-free alternative for the culture of fibroblasts in sufficient quantities to enable the production of feeder cell banks for clinical grade hESCs. (III)

- Cell culture, cryopreservation and feeder cell preparation protocols for clinical grade fibroblasts were optimized. (III)

4. The ECM production or growth factor secretion by the supporting feeder cells was associated with capacity to promote undifferentiated hESC culture and induce differentiation of hPSCs to RPE cells:

- Laminin-511 was verified to be produced by the hESC-supportive feeder types, hFFs and mEFs, but not by the hDF feeder cell lines, indicating a role in feeder-dependent hESC maintenance. Other genes with a possible role in feeder-dependent hESC support were additionally identified, namely vitronectin, E-cadherin and FGF regulator Anosmin-1. (III)

- Fibroblast secreted factors present in hFF-CM and mEF-CM enhanced early-stage hPSC-RPE cell differentiation. Higher activin A and TGF-ß1 secretion by the mEF and hFF cells respectively, were hypothesized as RPE inductive agents. Correspondingly, the addition of a low level of activin A to non-conditioned medium substantially enhanced hESC-RPE cell differentiation. (IV)

# Acknowledgements

This study was carried out at the Institute of Biomedical Technology (IBT) (formerly Regea - Institute for Regenerative Medicine), University of Tampere. Professor Riitta Seppänen MD, DDS, PhD is warmly thanked for providing excellent research facilities to work in. I want to thank Tampere Graduate Program in Biomedicine and Biotechnology for providing scientific education and financial support. Additionally, TEKES, the Finnish Funding Agency for Technology and Innovation, the Academy of Finland, the Finnish Cultural Foundation, the Competitive Research Funding of the Tampere University Hospital, the University of Tampere, the Orion-Farmos Research Foundation, and the City of Tampere are cordially thanked for financial support.

I want to express my profound gratitude to my supervisor Docent Heli Skottman, PhD, who gave me the opportunity to work in this project under her supervision. Thank you for all your effort and for always taking the time to help me with scientific challenges. I also want to thank my other supervisor Professor Outi Hovatta, MD, PhD for her help with the projects and the thesis. I greatly admire both of you for your scientific expertise and I am deeply grateful for all your help and encouragement during the years.

The members of my follow-up group, Docent Katriina Aalto-Setälä, MD, PhD and Professor Minna Kellomäki, D.Tech are warmly thanked for their valuable advice and encouragement in the yearly meetings. Docent Varpu Marjomäki, PhD and Professor Majlinda Lako, PhD are thanked for their constructive criticism and valuable comments on the thesis. I want to express my gratitude to my co-authors and collaborators Sarita Panula MSc, Suvi Aivio MSc, Professor Harri Pihlajamäki, Dr Sami Areva, Sanna Vuoristo MSc, late Professor Ismo Virtanen, Professor Timo Otonkoski, Marisa Ojala MSc, Katariina Maaninka MSc, Professor Seppo Auriola, Paediatric surgery specialist Dr Tarja Iber, Professor Petr Dvorak, Livie Eiselleova

PhD, Mika Pelto MSc (Tech) and Pipsa Kaipainen Head Technician for their contribution.

I want to thank the members of the eye group Tanja Ilmarinen, Kati Juuti-Uusitalo, Anni Sorkio and Alexandra Mikhailova for all your help and friendship. Our excellent laboratory staff Outi Melin, Hanna Koskenaho, Elina Kónsen and Niina Ikonen are most humbly thanked for all the technical assistance and for many cheerful moments in the lab. I want to thank all my workmates at Regea, especially Kristiina Rajala, Mari Pekkanen-Mattila and Riikka Äänismaa for their friendship and for all the sweat, tears, and most of all laughter during the years. Special thanks to wonderful Hanna Hiidenmaa whose friendship has meant the world to me.

My beloved friends Tytti, Jonna, Elina, Noora, Leena, Carita, and Satu. Thank you for the lasting friendships and for always being there for me. Maiju & Magnus, Jenni & Jani, thank you for the wonderful years in Uppsala, that were my springboard to pursuing a career in science and for staying in touch and in my life. And the slightly more recent friends in Mutikko: Susanna & Kari, Susanna & Timo, Heidi & Sakari are thanked for their friendship, for all the cheerful moments and for counterbalancing my life.

I want to acknowledge my family Mailis, Leif, Harri, Heli and Heta Hakala. My parents Mailis and Leif cannot be thanked enough. Thank you for always encouraging me and for all your invaluable help throughout the years. Mom's persistent help babysitting Fanny made it possible for me to finalize the thesis. And finally, I dedicate this thesis and express my sincerest gratitude to my daughter Fanny and my husband Arto, the most precious people in my life. Arto is admired and thanked for his patience, which undoubtedly was tested during this process. Thank you for your love that makes it all worthwhile. You mean everything to me.

Kangasala, November 2012

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# Original publications

Human Reproduction pp. 1–8, 2007 Hum. Reprod. Advance Access published January 24, 2007 doi:10.1093/humrep/del523

# Testing of nine different xeno-free culture media for human embryonic stem cell cultures

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**BACKGROUND: Human embryonic stem cells (hESC) are excellent candidates for cell replacement therapies. However, currently used culture conditions contain animal-derived components that bear a risk of transmitting animal pathogens and incorporation of non-human immunogenic molecules to hESC. METHODS: Nine xeno-free culture media were compared with the conventional serum replacement (ko-SR) containing media in the culture of hESC on human feeder cells. Cultured hESC were characterized immunocytochemically and by fluorescence-activated cell sorter analysis. The differentiation potential of hESC cultured with xeno-free media was determined with the RT–PCR analysis. RESULTS: The hESC cultured in xeno-free media differentiated or the proliferation decreased substantially. Under some test conditions, the morphology of the feeder cells was altered considerably. The hESC cultured with human serum underwent excessive differentiation in the beginning of culture, but a fraction of hESC was able to adapt to culture conditions containing 20% of human serum. CONCLUSIONS: None of the studied xeno-free media was able to maintain the undifferentiated growth of hESC. The medium containing 20% human serum was found to sustain undifferentiated hESC proliferation to some extent, yet was inferior to the conventional ko-SR-containing medium.**

*Key words*: human embryonic stem cell/human serum/xeno-free culture conditions

## Introduction

The first human embryonic stem cell (hESC) lines were derived and cultured on mitotically inactivated mouse embryonic fibroblast (MEF) cell layer using a medium containing fetal bovine serum (FBS) (Thomson *et al.*, 1998). Under the FBS-containing conditions, many cells died and the hESC colonies underwent excessive differentiation (Amit *et al.*, 2000; Reubinoff *et al.*, 2000; Amit and Itskovitz-Eldor, 2002). Amit *et al.* (2000) described serum-free culture conditions for hESC, using a commercial serum replacement (KnockOut-Serum Replacement, ko-SR, Invitrogen) instead of FBS in the hESC culture medium. It was shown that ko-SR supplemented with basic fibroblast growth factor (bFGF) was able to support a prolonged growth of hESC in an undifferentiated state, and a higher cloning efficiency was obtained than in the FBS-containing medium (Amit *et al.*, 2000). We have systematically compared different ko-SR concentrations for culturing hESC and found 20% to be the optimal concentration for the undifferentiated propagation of hESC derived and propagated on human feeder cells (Koivisto *et al.*, 2004). The use of ko-SR in the hESC culture medium provides more standardized culture conditions compared with FBS, which is highly variable from lot to lot. Currently, ko-SR has mostly replaced the use of FBS in hESC cultures in most laboratories. Unfortunately, ko-SR still contains animal proteins such as bovine serum albumin (BSA) and hence is not completely free of xeno-derived components (Price *et al.*, 1998). Human serum has also been used to replace FBS in hESC culture with some success. Richards *et al.* (2002) and Ellerström *et al.* (2006) derived hESC lines using culture medium containing human serum. Ellerstöm *et al.* managed to propagate hESC line in the human-serum-containing culture medium in an undifferentiated state for over 20 passages.

The exposure of hESC to xeno-products such as animal sera or proteins risks the contamination of hESC with undefined retroviruses and other animal pathogens (Amit *et al.*, 2003). In addition, certain animal molecules such as the sialic acid Neu5Gc are incorporated and expressed on hESC cultured in the presence of animal-derived products. Such non-human antigens result in an immune response in humans (Martin *et al.*, 2005). The use of MEF feeder cells in hESC culture is

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another major concern when aiming at developing xeno-free culture conditions for hESC. Various types of human feeder cells have been successfully used in maintaining hESC cultures (Richards *et al.*, 2002). Richards *et al.* (2003) showed human adult skin fibroblasts to be the best feeder cell type in a comparative evaluation of 11 different human adult, fetal and neonatal fibroblast feeder types. Our group has derived and cultured hESC lines using commercial human foreskin fibroblasts as feeder cells since 2002 (Hovatta *et al.*, 2003; Inzunza *et al.*, 2005).

Various feeder-free culture conditions have also been reported for the culture of hESC (Xu *et al.*, 2001; Amit *et al.*, 2004; Beattie *et al.*, 2005; Klimanskaya *et al.*, 2005; Stojkovic *et al.*, 2005). Some of these methods have been xeno-free by containing recombinant or human-derived extracellular matrixes and xeno-free media. A few hESC culture studies have been reported with X-Vivo 10 medium that contains only human-sourced recombinant proteins supplemented with recombinant human bFGF, stem cell factor, recombinant human flt3 ligand and leukaemia inhibitory factor (LIF) (Li *et al.*, 2005) or a high concentration of bFGF (Genbacev *et al.*, 2005). Recently, Ludwig *et al.* (2006) described a feeder-free derivation and culture of hESC using defined medium (TeSR1) including protein components solely from recombinant sources or purified from human material. However, feeder-free culture methods may induce chromosomal abnormalities in hESC due to the adaptation to more demanding growth conditions and enzymatic passaging methods often utilized in the feeder-free cultivation of hESC (Draper *et al.*, 2004; Mitalipova *et al.*, 2005). In feeder-free conditions, the importance of high concentrations of exogenously added growth factors and other factors increases. The heterogeneity of culture conditions and the variety and high concentrations of growth factors tested in sustaining undifferentiated growth of hESC reflect the fact that knowledge about the maintenance of self-renewal and pluripotency of hESC is still inadequate.

The use of xeno-derived components in the culture of hESC essentially limits the future clinical use of hESC-based therapies, and finding alternatives to replace these xeno-derived components has been one of the major focuses of hESC research during the past few years. Some commercial xeno-free serum replacements and media have recently become available. In order to find optimal culture conditions with low concentrations of bFGF using post-natal foreskin fibroblasts as feeder cells, we systematically tested eight commercially available or published xeno-free media and human serum in the culture of more than one hESC line.

## Materials and methods

### *Culture media*

Two commercial culture media, X-Vivo 10 and X-Vivo 20 (both from Cambrex Bio Science, Walkersville, MD, USA), published TeSR1 medium (Ludwig *et al.*, 2006; Thomson and Ludwig, 2006) with modifications, five commercially available serum replacements—Lipumin™ 10×, SerEx 10× (both from PAA Laboratories GmbH, Pasching, Austria), SR3 (Sigma, St Louis, MO, USA), serum substitute supplement (SSS) (Irvine Scientific, Santa Ana, CA, USA) and Plasmanate (Bayer Healthcare, West Haven, CT, USA)—and human serum (Sigma) were tested in the culture of hESC, as shown in Table I.

The control hESC medium contained 80% (vol/vol) KnockOut Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA, USA) and 20% (vol/vol) ko-SR (Invitrogen) supplemented with 2 mM Glutamax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 0.1 mM MEM non-essential amino acids (Cambrex Bio Science), 50 U penicillin/ml–50 μg streptomycin/ml (Cambrex Bio Science) and recombinant human bFGF (R&D Systems, Minneapolis, MN, USA) at 8 ng/ml. For feeder-free culture experiments, the hESC medium was supplemented with 80 ng/ml of human bFGF.

Xeno-free serum replacements Lipumin™ 10×, SerEx 10×, SR3 and SSS were tested at 10 and 20% concentrations and Plasmanate at 20 and 40% concentrations in KnockOut DMEM supplemented as the control hESC medium without ko-SR. X-Vivo 10 medium contained X-Vivo 10 basal medium and 0.12 ng/ml transforming growth factor β1 (TGFβ1, Sigma) and was supplemented as the control hESC medium without ko-SR. X-Vivo 20 medium contained X-Vivo 20 basal medium and was supplemented as the control hESC medium without ko-SR. The human-serum-containing culture medium contained KnockOut DMEM, 10 or 20% (vol/vol) of heat-inactivated, sterile filtered human serum (H1388, Sigma) and 50 mg/l L-ascorbic acid 2-phosphate and was supplemented as the control hESC medium without ko-SR. For feeder-free culture experiments, 20% human-serum-containing culture medium was supplemented with 80 ng/ml of human bFGF.

The modified TeSR1 medium contained DMEM/F12 basal medium (Invitrogen) supplemented with 16.5 mg/ml human serum albumin (Sigma), 196 μg/ml insulin (Invitrogen), 108 μg/ml human holo-transferrin (Sigma), 1:500 chemically defined lipid concentrate (Invitrogen), 2 mg/l reduced glutathione (Sigma), 1:1000 trace elements B and C solution (Cellgro, Herndon, VA, USA), 6 mg/l thiamine hydrochloride (Sigma), 0.02 mg/l sodium selenite (Sigma), 41.5 mg/l lithium chloride (Sigma), 0.1 mg/ml γ-aminobutyric acid (Sigma), 0.127 μg/ml pipecolic acid (Sigma), 0.6 ng/ml TGFβ1 (Sigma) and 50 mg/l L-ascorbic acid 2-phosphate (Sigma) supplemented as the control hESC medium without ko-SR. The modification made to the published TeSR1 medium (Ludwig *et al.*, 2006; Thomson and Ludwig, 2006) was the use of 8 ng/ml of bFGF in the experiments with human foreskin fibroblast feeder cells. Our previous testing has showed that there is no improved effect of higher concentration of bFGF for the growth of undifferentiated hESC in the presence of human foreskin fibroblasts (unpublished results); hence low concentration of bFGF was used for the experiments performed with human foreskin fibroblast feeder cells. For feeder-free culture experiments, the TeSR1 medium was supplemented with 100 ng/ml of human bFGF.

### *hESC cultures using human foreskin fibroblast feeder layer*

Human ESC lines HS181 (passages 60 and 62), HS237 (passages 59, 61 and 74), HS293 (passages 42 and 49) and HS306 (passage 50) derived at the Karolinska Institute, Stockholm, Sweden, were used for the culture experiments. The Karolinska Institute has an approval of the Ethics Committee of the Karolinska Institute for derivation, characterization and differentiation of hESC lines. REGEA, Institute for Regenerative Medicine, University of Tampere, Finland, has the approval of the Ethical Committee of Pirkanmaa Hospital District to culture hESC lines derived at the Karolinska Institute. These cell lines have been derived and cultured on human foreskin fibroblasts as feeder cells, and the lines have been characterized earlier (Hovatta *et al.*, 2003; Inzunza *et al.*, 2005). Commercially available human foreskin fibroblast cells (CRL-2429, ATCC, Manassas, VA, USA) were used as feeder cells for the culture of hESC. Before plating the hESC, the feeder cells were

**Table I.** The tested culture media for human embryonic stem cells (hESC)

| Test reagent | Cell line and passage | Medium composition[a] |
|---|---|---|
| Control hESC medium | HS181 p62<br>HS237 p59, p74<br>HS293 p49<br>HS306 p50 | 80% ko-DMEM; 20% ko-SR |
| Human serum | HS237 p74<br>HS306 p50 | 80/90% ko-DMEM; 10/20% human serum;<br>50 mg/l L-ascorbic acid 2-phosphate |
| Lipumin™ 10× | HS181 p62<br>HS237 p59, p74<br>HS293 p49 | 80/90% ko-DMEM; 10/20% Lipumin |
| Plasmanate | HS181 p62<br>HS237 p74 | 80/60% ko-DMEM; 20/40% Plasmanate |
| SerEx 10× | HS181 p62<br>HS237 p59<br>HS293 p49 | 80/90% ko-DMEM; 10/20% SerEx |
| Serum substitute supplement (SSS) | HS181 p62<br>HS237 p59, p74<br>HS293 p49 | 80/90% ko-DMEM; 10/20% SSS |
| SR3 | HS181 p60<br>HS237 p61<br>HS293 p42<br>HS237 p74 | 80/90% ko-DMEM; 10/20% SR3 |
| TeSR1 | HS181 p62 | DMEM/F12; 16.5 mg/ml HSA; 108 μg/ml holo-transferrin; 196 μg/ml insulin; 6 mg/l thiamine HCl; 41.5 mg/l LiCl; 2 mg/l reduced glutathione; 50 mg/l L-ascorbic acid; 1:1000 trace elements B and C solution; 0.1 mg/ml GABA; 0.02 mg/l sodium selenite; 0.127 μg/ml pipecolic acid; 0.6 ng/ml TGFβ1; 1:500 chemically defined lipid concentrate |
| X-Vivo 10 | HS237 p59<br>HS293 p49 | 100% X-vivo 10; 0.12 ng/ml TGFβ1 |
| X-Vivo 20 | HS181 p60<br>HS237 p61 | 100% X-vivo20 |

Ko-DMEM, KnockOut Dulbecco's modified Eagle's medium; ko-SR, KnockOut serum replacement; DMEM/F12, Dulbecco's modified Eagle's medium: F12 nutrient mixture; HSA, human serum albumin; LiCl, lithium chloride; GABA, γ-aminobutyric acid; TGFβ1, transforming growth factor β1.
[a]In all cases, test medium is supplemented with 2 mM Glutamax, 0.1 mM β-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 50 U penicillin/ml–50 μg streptomycin/ml, and 8 ng/ml basic fibroblast growth factor.

mitotically inactivated by irradiating with 40 Gy. The hESC were adapted to the test culture conditions by gradually increasing the concentration of the test medium and decreasing the concentration of the control hESC culture medium every second day during the adaptation phase. The hESC were cultured in a humidified +37°C, 5% $CO_2$ incubator. The growth of hESC was monitored microscopically and culture media were changed daily. The hESC cultures were passaged mechanically every 7–10 days to new feeder cells. Every test media experiment was performed with at least two different hESC lines. The experiments failing to maintain hESC undifferentiated were repeated for second time in order to verify the results of the first experiments.

### *Feeder-free culture of hESC*

Human ESC line HS237 (passage 78) was used for the feeder-free culture experiments. The hESC were adapted to the TeSR1 and 20% human-serum-containing media on human feeder cells by gradually increasing the concentration of the test medium and decreasing the concentration of the control hESC culture medium every second day during the adaptation phase. After adaptation phase, hESC were plated onto 12-well plates (CellBIND Surface, Corning, Inc., Corning, NY, USA) containing 10 μg/cm$^2$ human collagen IV (Sigma), 0.2 μg/cm$^2$ human vitronectin (Sigma), 5 μg/cm$^2$ human fibronectin (Sigma) and 5 μg/cm$^2$ human laminin (Sigma) coating mixture. The hESC were cultured in a humidified +37°C, 5% $CO_2$ incubator. The growth of hESC was monitored microscopically and culture media were changed daily. The feeder-free hESC cultures were passaged mechanically every 7–10 days and plated onto a new 12-well plate containing the coating mixture. The experiments which failed to maintain hESC undifferentiated were repeated for a second time in order to verify the results of the first experiments.

### *Immunofluoresence for Nanog and stage-specific embryonic antigen-1*

The hESC colonies were fixed in culture dishes with 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for 20 min at room temperature (RT), followed by washing with PBS (2 × 5 min). The cells were permeabilized and blocked with 0.1% Triton X-100, 1% BSA (Sigma) and 10% normal donkey serum (Sigma) in PBS for 45 min at RT and then washed once with 0.1% Triton X-100, 1% BSA and 1% normal donkey serum in PBS. Primary antibodies, polyclonal goat anti-human Nanog at a dilution of 1:200 and monoclonal mouse anti-human stage-specific embryonic antigen-1 (SSEA-1) at 1:200 (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. Primary antibodies were incubated overnight at 4°C. The cells were washed (3 × 5 min) with 1% BSA in PBS and probed with secondary antibodies: rhodamine-red-conjugated donkey anti-mouse immunoglobulin (Ig) M at 1:400 (Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK) and Alexa Fluor 488 donkey anti-goat IgG at 1:800 (Invitrogen) for 1 h in the dark at RT. Human ESC labelled only with secondary antibodies were used as negative controls. After incubation, the cells were washed with PBS (3 × 5 min) and mounted in Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA, USA). Human ESC line HS237 cultured with the hESC medium was used as a control in immunofluoresence analysis. The labelled cells were viewed and

photographed with a Nikon Eclipse TE2000-S phase contrast microscope with fluorescence optics and a Nikon COOLPIX 5400 camera.

### *Fluorescence-activated cell sorter analysis*

The hESC (HS237) cultured in medium containing 20% human serum were analysed using a fluorescence-activated cell sorter (FACS). The cells were dissociated from the culture dish with Tryple™ Select (Invitrogen) for 15 min at 37°C and resuspended in 1 ml FACS buffer I (2% FBS, 0.01% sodium azide in PBS) and counted with a haemacytometer. A total of $0.2 \times 10^6$ cells were recovered, half of which were probed for 15 min at 4°C with a 1:500 dilution of monoclonal mouse anti-human SSEA-4 (Santa Cruz Biotechnology, Inc.) and the other half with monoclonal mouse anti-human SSEA-1 in FACS buffer I. The cells were then washed with FACS buffer I and probed with FACS buffer I containing a 1:500 dilution of r-phycoerythrin-conjugated goat anti-mouse IgG or r-phycoerythrin-conjugated goat anti-mouse IgM (both from Invitrogen) for 15 min in the dark at 4°C. The cells were then washed once with FACS buffer I, once with FACS buffer II (0.01% sodium azide in PBS) and fixed with 1% formaldehyde in PBS. HS237 cells cultured in a hESC medium were used as a control and treated similarly. The samples were analysed using BD FACSAria™ equipment (BD Biosciences, Franklin Lakes, NJ, USA). Acquisition was set for 10 000 events per sample. The data were analysed using FACSDiva Software version 4.1.2.

### *In vitro differentiation and RT–PCR analysis*

The pluripotency of the hESC line HS237 cultured with the modified TeSR1 medium was analysed with the RT–PCR analysis. The embryoid bodies (EB) were formed by mechanically dissecting upward-growing hESC colonies at passage 7 and transferring the resulting pieces onto a culture dish without feeder cells. The EBs were cultured in a modified TeSR1 medium without bFGF for 23 days before the isolation of RNA. The medium was changed every 2–3 days. The hESC line HS181 cultured in a hESC medium was used as a control, and samples were prepared similarly. Total RNA was isolated from EBs (TeSR1, $n = 25$; control hESC medium, $n = 5$) using RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNA extraction was performed according to the manufacturer's instructions. The concentration and quality of isolated RNA were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Complementary DNA (cDNA) was synthesized from 50 ng of total RNA using Sensiscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The expression of markers characteristic of ectoderm (neurofilament 68 kDa, sense 5′-GAG TGA AAT GGC ACG ATA CCT A-3′; antisense 5′-TTT CCT CTC CTT CTT CAC CTT C-3′), endoderm (α-fetoprotein, sense 5′-GCT GGA TTG TCT GCA GGA TGG GGA A-3′; antisense 5′-TCC CCT GAA GAA AAT TGG TTA AAA T-3′) and mesoderm (α-cardiac actin, sense 5′-GGA GTT ATG GTG GGT ATG GGT C-3′; antisense 5′-AGT GGT GAC AAA GGA GTA GCC A-3′) development in EBs were determined using RT–PCR primers (Proligo, Sigma). Glyceraldehyde 3-phosphate dehydrogenase (sense 5′-AGC CAC ATC GCT CAG ACA CC-3′; antisense 3′-GTA CTC AGC GGC CAG CAT CG-5′) was used as a housekeeping control. One microlitre of cDNA was used as template in the PCR reactions. The negative control contained sterilized water instead of cDNA template. The PCR reactions were carried out in the Eppendorf Mastercycler as follows: denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The PCR products were analysed with electrophoresis on 1.5% agarose gel containing 0.4 μg/ml ethidium bromide (Sigma) and DNA standard (MassRulerTM DNA Ladder Mix, Fermentas).

## Results

Human ESC were gradually adapted to different test media, using an increasing proportion of test media (with ratios of test media to control hESC media at 20:80, 50:50 and 80:20) up to 100% during the first week of culture. Different concentrations of commercially available serum replacements and media were used to evaluate the growth and maintenance of undifferentiated hESC. None of the eight xeno-free culture media or serum replacements tested were able to maintain the undifferentiated growth of hESC on human feeder cells. The differentiation of hESC already began during the adaptation process with all test media, as indicated by the change in colony morphology. The colonies became thinner and lost their angular shape and sharp edges. The number of undifferentiated colonies diminished significantly after the third adaptation phase (80:20) at all concentrations (10 and 20%), tested with Lipumin™-, SerEx-, SSS-, SR3-, X-Vivo-10- (100%), X-Vivo-20- (100%) and Plasmanate- (20 and 40%) containing media.

The results were consistent in all hESC lines examined and in repeated experiments. The differentiation was first judged by morphology and then confirmed by immunofluoresence analysis. The hESC colonies grown with the test culture media in all tested concentrations showed an increased expression of a marker common to the differentiated hESC (SSEA-1) and were negative for a marker common to the undifferentiated hESC (Nanog) (Figure 1).

The morphology of the feeder cells used was found to change under some test conditions. Feeder cells shortened, became spherical and started to detach in X-Vivo-10-, X-Vivo-20-, SR3- and Lipumin™-containing media (Figure 1). In some test conditions, the growth of the colonies was also reduced significantly (X-Vivo 10, SSS, SerEx, modified TeSR1). Human ESC were all differentiated (except in modified TeSR1 media) when the adaptation process was complete, and it was impossible to passage the colonies further. The modified TeSR1 media were able to maintain the undifferentiated growth of hESC on feeder cells for seven passages. The hESC colonies in modified TeSR1 media began to grow upwards after seven passages, and the experiment was aborted.

The upward-growing hESC colonies cultured in the modified TeSR1 medium using feeder cells were differentiated *in vitro* into EBs, which were analysed with RT–PCR. Ectoderm (neurofilament 68 kDa) and mesoderm (α-cardiac actin) specific markers were detected in the RT–PCR analysis. However, an endoderm (α-fetoprotein) specific marker was not detected (Figure 2). These results show a defective pluripotency of hESC cultured with the modified TeSR1 medium. Our results clearly show that the various xeno-free test media were not able to maintain the undifferentiated growth and the pluripotency of hESC on human feeder cells.

Human ESC were gradually adapted to human-serum-containing media using an increasing proportion of test media. The human-serum-containing medium was tested with

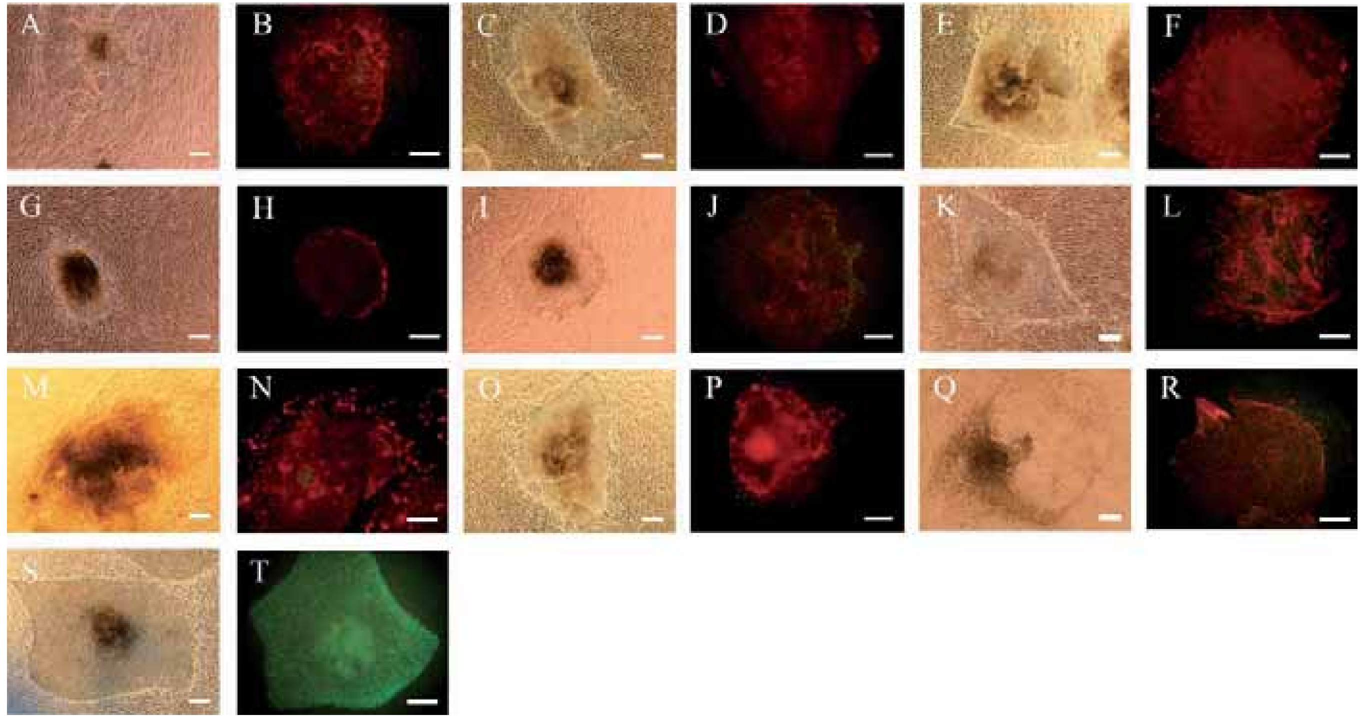


**Figure 1.** The morphology and immunofluoresence analyses of the human embryonic stem cell (hESC) colonies cultured with eight different xeno-free media. (**A**) An hESC colony after one passage (20% Lipumin); (**B**) the expression of Nanog (green) and stage-specific embryonic antigen-1 (SSEA-1) (red) after one passage (20% Lipumin); (**C**) an hESC colony after one passage (20% Plasmanate); (**D**) the expression of Nanog (green) and SSEA-1 (red) after one passage (20% Plasmanate); (**E**) an hESC colony after one passage (40% Plasmanate); (**F**) the expression of Nanog (green) and SSEA-1 (red) after one passage (40% Plasmanate); (**G**) an hESC colony after one passage (20% SerEx); (**H**) the expression of Nanog (green) and SSEA-1 (red) after one passage (20% SerEx); (**I**) an hESC colony after one passage (20% SR3); (**J**) the expression of Nanog (green) and SSEA-1 (red) after one passage (20% SR3); (**K**) an hESC colony after one passage [20% serum substitute supplement (SSS)]; (**L**) the expression of Nanog (green) and SSEA-1 (red) after one passage (20% SSS); (**M**) an hESC colony after one passage (X-Vivo 10); (**N**) the expression of Nanog (green) and SSEA-1 (red) after one passage (X-Vivo10); (**O**) an hESC colony after one passage (X-Vivo 20); (**P**) the expression of Nanog (green) and SSEA-1 (red) after one passage (X-Vivo 20); (**Q**) an hESC colony after seven passages (TeSR1); (**R**) the expression of Nanog (green) and SSEA-1 (red) after seven passages (TeSR1); (**S**) an hESC colony cultured with control hESC medium; (**T**) the expression of Nanog (green) and SSEA-1 (red) of hESC colony cultured with control hESC medium. Scale bar-200 μm.

concentrations of 10 and 20% of heat-inactivated, sterile filtered human serum. The hESC colonies underwent excessive differentiation during the first passages, as the concentration of human serum was gradually increased and the concentration of ko-SR in the culture medium was decreased. The differentiation was indicated by the change of hESC colony morphology. The colonies got thinner and some lost their angular shape and defined borders. The medium containing 10% of human serum was able to maintain the undifferentiated hESC growth for nine passages on human feeder cells. At the end of passage 10, all colonies had differentiated completely and the differentiation was confirmed by immunofluoresence staining with Nanog and SSEA-1 (Figure 3). As the colonies in the culture medium containing 20% of human serum were passaged further, they began to regain their thicker, undifferentiated morphology at passage level 8. At passage level 11, the colonies showed undifferentiated morphology (Figure 3), although they were notably thinner than the hESC cultured in the presence of a control hESC culture medium (Figure 3). At passage level 11, the hESC cultured in the 20% human serum medium were stained with a panel of immunocytochemical antibodies specific to hESC markers: Nanog, OCT-3/4, SSEA-4 and SSEA-1 (Figure 3). The expression of the markers was not complete, and parts of the colonies were differentiated, although there was no expression of SSEA-1 (Figure 3). As a control, hESC cultured in a control hESC medium were stained with

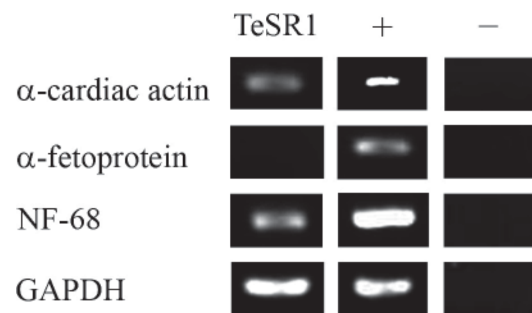


**Figure 2.** RT−PCR analysis of embryoid bodies (EBs) differentiated from the hESC line HS237 cultured with the modified TeSR1 medium. EBs cultured with TeSR1 medium expressed markers for the two embryonic germ cell layers, ectoderm [neurofilament (NF)-68] and mesoderm (α-cardiac actin). Endoderm (α-fetoprotein) specific marker was not detected (TeSR1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control. EBs differentiated from the hESC line HS181 cultured with control hESC medium were used as controls (+). Water was used as negative control ( − ).

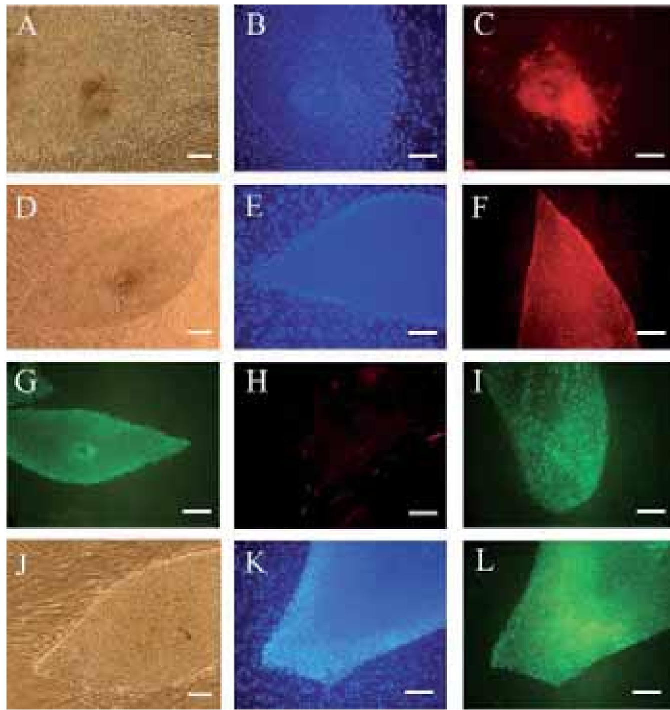


**Figure 3.** The morphology and immunofluoresence analyses of the hESC colonies cultured with human-serum-containing media. The hESC cultured with 10% human-serum-containing medium for 10 passages; (**A**) a differentiated hESC colony; (**B**) 4′,6-diamidino-2-phenylindole (DAPI) staining; (**C**) an hESC colony double stained with Nanog (green) and SSEA-1 (red). The hESC cultured with 20% human-serum-containing medium for 11 passages; (**D**) an hESC colony cultured with 20% human serum; (**E**) DAPI staining; (**F**) an hESC colony stained with SSEA-4; (**G**) an hESC colony stained with Oct − (3/4); (**H**) an hESC colony stained with SSEA-1; (**I**) an hESC colony stained with Nanog. The hESC cultured with control hESC medium; (**J**) an hESC colony cultured with control hESC medium; (**K**) DAPI staining; (**L**) an hESC colony double stained with Nanog (green) and SSEA-1 (red). Scale bar-200 μm.

Nanog and SSEA-1 and showed a strong expression of Nanog and no expression of SSEA-1 (Figure 3).

Human ESC cultured with 20% human-serum-containing medium on human feeder cells were further analysed with an FACS. In total, $0.1 \times 10^6$ cells were labelled with SSEA-4 and $0.1 \times 10^6$ with SSEA-1. According to the FACS analysis, 35% of the hESC were positive for SSEA-4 (Figure 4). Of the control hESC, 80% were positive for SSEA-4. The 20% human serum culture medium was found to sustain an undifferentiated hESC proliferation to some extent, however, being inferior to the currently used culture medium containing ko-SR.

The control hESC medium, TeSR1 medium and a medium containing 20% human serum were used to evaluate the growth and maintenance of undifferentiated hESC on extra-cellular matrix coating mixture without human foreskin fibro-blast feeder cells. The attachment of hESC was poor; from the hESC colony pieces which were plated with each medium, only 30% attached in the TeSR1 medium, 55% in 20% human-serum-containing medium and 68% in the control hESC medium. From the colony pieces attached, only 30% formed colonies in the TeSR1 medium, whereas 75%

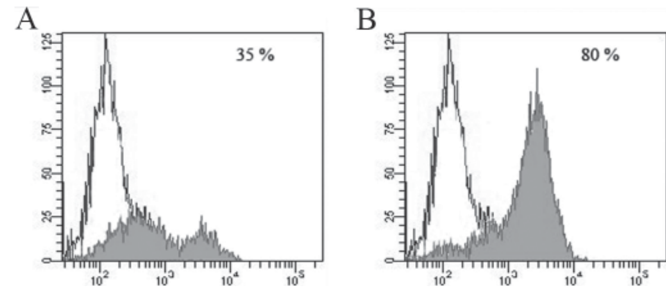


**Figure 4.** Fluorescence-activated cell sorter analysis of the hESC cultured with 20% human-serum-containing medium on human feeder layer for 11 passages. The percentage of positive cells is shown. (**A**) Surface-marker SSEA-4 expression of the hESC cultured with 20% human serum; (**B**) surface-marker SSEA-4 expression of the hESC cultured with control hESC medium.

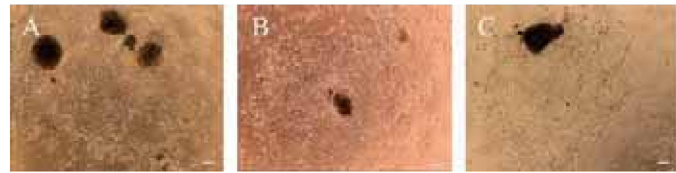


**Figure 5.** The morphology of hESC colonies cultured in feeder-free conditions at passage 1. (**A**) hESC cultured with control hESC medium; (**B**) hESC cultured with 20% human-serum-containing medium; (**C**) hESC cultured with TeSR1 medium. Scale bar-200 μm.

formed colonies in the 20% human-serum-containing medium and hESC medium (Figure 5). After second passaging, only minor colony formation was observed in the hESC control medium and in 20% human-serum-containing medium, whereas no colony formation was observed in the TeSR1 medium and the experiment was terminated.

## Discussion

In this study, nine commercially available or published xeno-free media and serum replacements were compared with the conventionally used serum replacement (ko-SR) containing medium in the culture of hESC. These xeno-free reagents were not able to maintain the undifferentiated growth of hESC. The cell proliferation decreased during the adaptation to the test media, and after the complete adaptation, the hESC quickly differentiated. The use of commercially available X-Vivo 10 medium has been previously described by two research groups (Genbacev *et al.*, 2005; Li *et al.*, 2005) in feeder-free culture conditions of hESC. Li *et al.* supplemented the X-Vivo 10 medium with recombinant human bFGF, stem cell factor, recombinant human flt3 ligand and LIF, whereas Genbacev *et al.* used a high concentration of bFGF. Recently, Ludwig *et al.* (2006) reported feeder-free derivation and culture of hESC using defined medium (TeSR1) including protein components solely from recombinant sources or purified from human material. In addition, they used a combination of collagen IV, fibronectin, laminin and vitronectin coating from human sources instead of a feeder cell layer. They managed to derive two hESC lines in these animal-product-free con-ditions, although neither one maintained stable karyotype. The

TeSR1 medium contains various ingredients, of which many are used in high concentrations, making the medium very expensive. Also, the purified human matrix components used in the coating are expensive. Unfortunately, neither X-Vivo 10 medium nor TeSR1 with low concentration of bFGF were able to sustain the undifferentiated growth of our hESC lines on human foreskin feeder layer. The proliferation of hESC cultured in the modified TeSR1 medium decreased significantly after the adaptation process. Human ESC colonies began to grow upwards, making the passaging impossible. The modified TeSR1 media were able to maintain the undifferentiated growth of hESC for seven passages on human feeder cells. However, the pluripotency of hESC was incomplete, as an endoderm-specific marker was not detected in the RT–PCR analysis of the hESC cultured in the modified TeSR1 medium. We also tested feeder-free cultivation of hESC in the TeSR1 medium. However, the attachment and colony formation in the TeSR1 medium was poor compared with the control hESC medium, and we were able to maintain hESC in the TeSR1 medium only for two passages.

The morphology of the feeder cells used was found to change under some test conditions. Feeder cells shortened, became spherical and started to detach in X-Vivo-10-, X-Vivo-20-, SR3- and Lipumin™-containing media. Because feeder cells are an important part of our culture system, this might have a critical effect on the differentiation of hESC. It is reported that ascorbic acid deficiency in a fibroblast culture causes, among other things, an easy disaggregation of the cells from the intracellular matrix by protease action (Schafer *et al.*, 1967). The role of ascorbic acid in a cell culture is to function as an antioxidant for the cells. Ascorbic acid is not available in a standard basal culture medium and needs to be added to the medium as a stable phosphate (Geesin *et al.*, 1993). The ko-SR (Gibco Invitrogen) contains ascorbic acid (Price *et al.*, 1998), but it is not known whether the commercially available serum replacements and media tested contain L-ascorbic acid. The absence or presence of other components in the tested culture media may also have influenced the decreased proliferation and differentiation of hESC. It is certain that the ingredients of the control and the test culture media differ because once the control media is entirely replaced, the differentiation of hESC is excessive. Our results clearly show that the eight different culture media tested were not able to support the undifferentiated growth of hESC. These results suggest that unknown components either present in or absent from the tested media compared with ko-SR induce the differentiation of hESC.

Human serum has previously been used in hESC culture with some success. Richards *et al.* (2002) derived an hESC line using 20% human-serum-containing culture medium and were able to propagate hESC in an undifferentiated state for 10 passages. However, it was later observed that a prolonged use of human serum beyond the 10th passage led to the increased differentiation of hESC (Richards *et al.*, 2003). Recently, Ellerström *et al.* were able to derive and propagate an hESC line in the human-serum-containing medium for over 20 passages, without problems of excessive differentiation. In our study, human serum medium (containing L-ascorbic acid and 20% of human serum) was found to maintain undifferentiated hESC growth on human feeder cells to some extent (11 passages), but the hESC underwent an excessive differentiation in human serum culture media in the beginning of the experiments. As Richards *et al.* (2003) stated, human serum may contain some unknown factors that promote the differentiation of hESC, whereas ko-SR contains factors that enable an undifferentiated hESC culture. Since a fraction of the cells could be passaged despite the excessive differentiation in the beginning of the experiment and since the colony morphology improved after several passages in 20% human serum medium, the population of cells was able to adapt to culture conditions with human serum. The hESC cultured in media containing 10% of human serum were not able to adapt to these culture conditions. This is most likely due to a lack of sufficient serum proteins in the culture media. In particular, the amount of albumin, which is the major blood protein, seems to be essential for hESC survival *in vitro*. This notion is supported by the fact that BSA is the major constituent of ko-SR (Price *et al.*, 1998). Although human serum can provide nutritive supplementation for hESC, it is a complex mixture containing compounds both beneficial and detrimental to hESC, which means that each lot should be carefully tested prior to use.

The addition of L-ascorbic acid to the culture medium could play a role in the ability of a subpopulation of hESC to survive in the human-serum-containing culture medium. The hESC cultured in 20% human serum medium maintained their undifferentiated morphology with smooth, angular colony shape for at least 11 passages, even though the colonies were thinner when compared with those cultured in the control hESC medium. We also tested feeder-free culture of hESC in 20% human-serum-containing medium. The attachment and colony formation in the human-serum-containing medium was considerably better than that in the TeSR1 medium. We also observed that the morphology of the cells in the colonies was considerably different than that in the control hESC medium and TeSR1 medium.

The use of human serum in the hESC culture medium is a xeno-free alternative to ko-SR, but similar to FBS, human serum is not of defined composition and is thus suboptimal. The commercial human sera available are pooled from a heterogeneous group of donors. Pooling sera from selected donors might provide more homogenous sera for hESC culture. Yet another concern with the use of human serum is the potential transmission of extremely serious pathogens such as human immunodeficiency virus, which has a long latent preclinical period and could go undetected in routine donor screenings (Mallon *et al.*, 2006). The optimal solution for the xeno-problem of ko-SR would be the use of a similarly defined serum replacement containing purified human or human recombinant components.

The various culture conditions reported are difficult to compare because each group has used a different base media, matrix or feeders, cell lines and cell passage numbers. Although hESC have been shown to grow in each of these conditions, it is unclear which, if any, of these culture conditions are optimal. The use of a single substrate for hESC growth is

desirable, but the substrates used are still undefined components and may have a lot-to-lot variability (Hoffman and Carpenter, 2005). It is also unclear whether the hESC maintained in these different substrates are equivalent. In fact, the gene expression signature of hESC is reported to be different when cultured with the ko-SR-containing medium and FBS (Skottman *et al.*, 2006).

Even though several improvements in hESC culture conditions have been achieved during the past few years, a completely xeno-free, defined culture and derivation methods for hESC, which could be reproduced in various laboratories culturing hESC lines worldwide, have not been established. Cells used for human transplantation are regulated by the European Union (EU). According to new EU directives (2003/94/EC and 2004/24/EC), hESC for transplantation must be cultured according to good manufacturing practice (GMP) requirements. In order to derive clinical-grade hESC lines, the used derivation methods and all constituents of culture must be of GMP grade. Continuous research for identifying the mechanisms of self-renewal of hESC and improving the current culture conditions is essential in order to establish completely xeno-free culture systems that meet the requirements of GMP and enable the large-scale production of hESC.

## Acknowledgements

We thank the personnel of Regea, Institute for Regenerative Medicine, for their support in the stem cell research. This work was supported by the Academy of Finland, TEKES, the Finnish Funding Agency for Technology and Innovation, the Competitive Research Funding of the Pirkanmaa Hospital District, the Finnish Defence Forces and the Swedish Research Council.

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*Submitted on October 6, 2006; resubmitted on December 13, 2006; accepted on December 27, 2006*

TISSUE ENGINEERING: Part A
Volume 15, Number 7, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/ten.tea.2008.0316

# Comparison of Biomaterials and Extracellular Matrices as a Culture Platform for Multiple, Independently Derived Human Embryonic Stem Cell Lines

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Long-term *in vitro* culture of undifferentiated human embryonic stem cells (hESCs) traditionally requires a fibroblast feeder cell layer. Using feeder cells in hESC cultures is highly laborious and limits large-scale hESC production for potential application in regenerative medicine. Replacing feeder cells with defined human extracellular matrix (ECM) components or synthetic biomaterials would be ideal for large-scale production of clinical-grade hESCs. We tested and compared different feeder cell–free hESC culture methods based on different human ECM proteins, human and animal sera matrices, and a Matrigel™ matrix. Also selected biomaterials were tested for feeder cell–free propagation of undifferentiated hESCs. The matrices were tested together with conventional and modified hESC culture media, human foreskin fibroblast-conditioned culture medium, chemically defined medium, TeSR1, and modified TeSR1 media. The results showed the undefined, xenogeneic Matrigel to be a superior matrix for hESC culture compared with the purified human ECM proteins, serum matrices, and the biomaterials tested. A long-term, feeder cell–free culture system was successful on Matrigel in combination with mTeSR1 culture medium, but a xeno-free, fully defined, and reproducible feeder cell–free hESC culture method still remains to be developed.

## Introduction

**H**UMAN EMBRYONIC STEM CELLS (hESCs) are traditionally cultured *in vitro* on mitotically inactivated mouse embryonic fibroblast (MEF) or human neonatal or fetal fibroblasts feeder cell layers.[1–5] The function of the feeder cells in the hESC coculture system is still not fully understood. The feeder cells provide hESCs with appropriate cell–cell contacts and also secrete soluble factors necessary to maintain the undifferentiated hESC status. This coculture system, however, presents several challenges. The production of feeder cells is highly laborious and limits the large-scale production of hESCs for future clinical applications. Also, the risk of incorporating animal pathogens and immunogenic animal proteins into hESCs limits the use of xeno materials such as fetal bovine serum (FBS) commonly used for fibroblast feeder cell propagation.[6,7]

The development of feeder cell–free hESC culture conditions has been an important focus of recent hESC research. In 2001, Xu *et al*. described the first feeder cell–free hESC culture conditions using Matrigel™, a complex mouse sarcoma cell basement membrane extract comprising various extracellular matrix (ECM) proteins and growth factors, in combination with a culture medium conditioned by MEFs (MEF-CM).[8] Conditioning, that is, incubating the hESC culture medium on a layer of MEF feeder cells before using the medium in hESC culture, allows the fibroblasts to secrete the necessary growth and attachment factors into the culture medium. In addition, FBS coating has been used as a hESC culture matrix combined with a chemically defined culture medium (CDM), first for mouse ESC culture[9] and later for hESC culture.[10] Stojkovic *et al*. reported successful maintenance of undifferentiated hESCs on human serum (HS) coating together with culture medium conditioned by fibroblast-like cells derived from hESCs.[11] Such culture systems are important steps forward, but are still xenogeneic and undefined.

Various human ECM proteins in combination with a variety of more or less defined culture media have also been used. Amit and Itskovitz-Eldor described a feeder cell–free and serum-free hESC culture system on human fibronectin

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coating and culture medium containing Knock-Out Serum Replacement (ko-SR) together with transforming growth factor β (TGFβ) and basic fibroblast growth factor (bFGF).[12] Commercial ko-SR has mostly replaced FBS as an hESC culture medium supplement as it is a more defined, serum-free alternative but still contains animal proteins such as bovine serum albumin (BSA).[13] In 2006, Ludwig *et al*. published the first feeder cell–free and xeno-free derivation of two hESC lines using a combination of four human ECM proteins as an attachment matrix and a defined, xeno component–free culture medium called TeSR1.[14] Both of the derived hESC lines, however, were found to be karyotypically abnormal. A modified, more economical xeno protein–containing version of the medium (mTeSR1) combined with Matrigel matrix was reported and became commercially available later the same year.[15]

Human sourced or recombinant ECM components are very expensive and vary batch to batch, whereas a synthetic biomaterial would offer a fully defined, consistent hESC culture platform. Most of the work done so far on synthetic biomaterials and scaffolds for hESC culture has involved the promotion of differentiation[16] and transplantation applications as well as cell encapsulation strategies.[17,18] In addition, most of the studies have been performed using mouse or nonprimate ESCs[19–23]; thus, the results are not usable for hESCs due to the different cell characteristics and cell growth behavior between the species.[24] Recently, undifferentiated propagation of hESCs has been studied using different biomaterial substrates, but these studies involved only relatively short-term hESC culture, and the methods were based on the use of MEFs or MEF-CM in a coculture system.[25,26]

In the present study, we aimed to find a sustainable feeder cell–free hESC culture method by systematically testing and comparing selected culture methods reported by other research groups to support their hESC lines in the absence of a feeder layer. We chose methods based on human ECM proteins (i.e., collagen IV, vitronectin, fibronectin, and laminin), human and animal sera matrices, and Matrigel as

Table 1. Summary of the Feeder Cell–Free hESC Culture Methods Analyzed

| *Matrix/biomaterial* | *Medium* | *hESC line and passage used* | *Max passage* |
|---|---|---|---|
| Ti | hES/hES-CM | HS293 p42–p59 | 1 |
| $TiO_2$ | hES/hES-CM | HS293 p42–p59 | 1 |
| $ZrO_2$ | hES/hES-CM | HS293 p42–p59 | 1 |
| PDTEC | hES/hES-CM | HS293 p54 | 1 |
| | | HS237 p71 | 1 |
| PLDLA | hES/hES-CM | HS237 p63 | 1 |
| Fibronectin | mhES | HS360 p62 | 2 |
| | | HS401 p50 | 1 |
| | CDM | HS401 p40–p45 | 2 |
| | | HS360 p53–p56 | 2 |
| B&D BioCoat, | mhES | HS360 p61 | 2 |
| Human fibronectin | | HS401 p49 | 2 |
| cellware | CDM | HS360 p59–p61 | 2 |
| | | HS401 p49 | 2 |
| Human ECM mixture: | hES/hES-CM | HS237 p79 | 1/2 |
| Collagen IV | | HS360 p79 | 2/2 |
| Vitronectin | | HS401 p48 | 2/2 |
| Fibronectin | TeSR1 | HS360 p54–p56 | 6 |
| Laminin | | HS401 p41–p42 | 7 |
| HS coating | hES/hES-CM | HS293 p50–p51 | 1/1 |
| | | HS237 p62–p80 | 1/2 |
| | | HS401 p32–p35 | 1/3 |
| | CDM | HS401 p39 | 2 |
| | | HS360 p52 | 1 |
| | | H237 p94 (46X, abnormal X) | 14 (9 + 5) |
| FBS coating | CDM | HS360 p52–p57 | 10 |
| | | HS401 p39 | 3 |
| | | HS237 p94 (46X, abnormal X) | 13 |
| Matrigel | hES/hES-CM | HS401 p48 | 2/5 |
| | mTeSR1 | HS401 p48–p56 | >30 |
| | | Regea 06/015 p71 | >30 |

Ti, titanium; $TiO_2$, titanium dioxide–coated titanium; $ZrO_2$, zirconium dioxide–coated titanium; PDTEC, poly(desaminotyrosyl-tyrosine-ethyl ester carbonate); PLDLA, poly-L,D-lactide; ECM, extracellular matrix; HS, human serum; FBS, fetal bovine serum; hES, standard hESC culture medium consisting of ko-DMEM, 20% ko-SR, 2 mM GlutaMax, 1% nonessential amino acids, 0.5% penicillin/streptomycin, 0.1 mM β-mercaptoethanol, and 8 ng/mL bFGF; hES-CM, human foreskin fibroblast-conditioned hES medium; TeSR1, chemically defined xeno-free hESC culture medium; mTeSR1, modified TeSR1 containing xeno-derived components; CDM, chemically defined medium; mhES, modified hES medium: 15% ko-SR, 0.12 ng/mL TGFβ, and 4 ng/mL bFGF.

culture matrices, to seek the true potential of the various matrix–media combinations to support hESC attachment and growth. The matrices were tested together with conventional hESC culture medium (hES medium), modified hES (mhES) medium, hES medium conditioned with human foreskin fibroblasts (hES-CM), and three different chemically defined media (CDM, TeSR1, and mTeSR1).

In addition, selected biomaterials, pure titanium (Ti), titanium dioxide ($TiO_2$)– and zirconium dioxide ($ZrO_2$)–coated titanium, poly-L,D-lactide (PLDLA), and poly (desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTEC), were tested as hESC culture substrates. Ti and $TiO_2$-coated Ti have been used in tissue engineering applications as seeding scaffold for bone marrow stromal cells[27] and Ti dishes as culture substrate for mesenchymal stem cells with excellent attachment and proliferation.[28] The PLDLA and PDTEC were chosen as potential hESC culture substrates because polylactic acid has been used for hESC differentiation[29] and tyrosine-derived polycarbonate for guided bone regeneration in animal models.[30]

Many of the published feeder cell–free culture methods have not been verified to maintain undifferentiated hESC culture of different hESC lines. In addition, it has been suggested that feeder cell–free culture methods may even cause chromosomal abnormalities.[31] Thus, we used several independently derived hESC lines in our experiments as well as a karyotypically abnormal hESC line to evaluate the different hESC culture methods.

## Materials and Methods

The culture matrix and media combinations tested and the hESC lines and passages used are summarized in Table 1.

### *hESC lines and culture*

Five karyotypically normal hESC lines were used in the experiments: HS237 (46, XX), HS293 (46, XY), HS360 (46, XY), HS401 (46, XY), and Regea 06/015 (46, XY). In addition, karyotypically abnormal HS237 (46X, abnormal X) hESC line was used. All hESC lines except Regea 06/015 were derived at the Karolinska Institutet in Stockholm, Sweden, by Professor Outi Hovatta's research group and characterized as described previously.[1,32] The Regea 06/015 line was derived and characterized in our laboratory similarly to the other hESC lines. The Ethics Committee of Pirkanmaa Hospital District approved the study to culture the hESC lines used. All hESC lines were cultured on irradiated (40 Gy) human foreskin fibroblast (CRL-2429; American Type Culture Collection [ATCC], Manassas, VA) feeder cells (hFF) and using hESCs culture medium prior the transfer to feeder cell–free culture conditions. All hESC lines are regularly characterized for expression of markers of undifferentiated hESCs (Nanog, OCT-3/4, SSEA-3, SSEA-4, TRA-1-81, and TRA-1-60) by immunocytochemical stainings, pluripotency by embryoid body formation and RT-PCR for markers of the three embryonic germ layers, and karyotypic stability by standard G-banding. The typical morphology and hESC marker expression of undifferentiated hESCs on hFF feeder cells are shown in Figure 1.

The hESCs were passaged manually to all feeder cell–free culture systems tested, culture media were changed, and growth was monitored daily. The cells were passaged either manually or with a combination of manual and enzymatic techniques using 0.5–1 mg/mL dispase or 1–5 mg/mL collagenase IV (both from Invitrogen, Carlsbad, CA) every 3–7 days when the colonies reached an appropriate size without excessive differentiation.

### *Culture media*

**hES medium.** The conventional hESC culture medium (hES medium) consisted of ko-DMEM (Invitrogen) supplemented with 20% ko-SR (Invitrogen), 2 mM GlutaMax (Invitrogen), 1% MEM Eagle nonessential amino acid solution (Cambrex Bio Science, Walkersville, MD), 0.5% penicillin/streptomycin (Cambrex Bio Science), 0.1 mM β-mercaptoethanol (Invitrogen), and 8 ng/mL bFGF (R&D Systems, Minneapolis, MN).

**Human foreskin fibroblast-conditioned hES medium.** The hFF-conditioned hES-CM was produced by adding conventional hES medium to a culture dish containing irradiated (40 Gy) hFF cells as a confluent monolayer of approximately $4 \times 10^4$ cells/cm$^2$. The hES medium was incubated at 37°C,

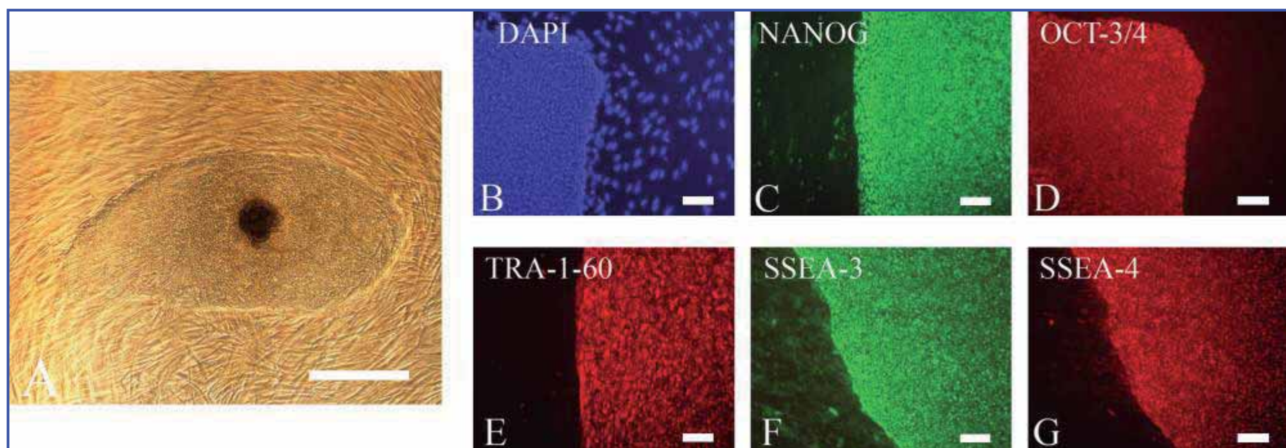


**FIG. 1.** Morphology of an undifferentiated colony of Regea 06/015 hESC line cultured in standard culture conditions on hFF feeders in hES medium, and the expression of markers of undifferentiated hESCs (Nanog, OCT-3/4, TRA-1-60, SSEA-3, and SSEA-4). Scale bars = 200 μm except for (**A**) = 500 μm.

5% $CO_2$ incubator for 24 h and thereafter used as culture medium for hESCs. An additional 8 ng/mL bFGF was freshly added to the CM before using in feeder cell–free hESCs culture, except for media used for the biomaterial testing.

Modified hES medium. The modified hES medium (mhES) was prepared as described by Amit and Itskovitz-Eldor.[12] Modified hES medium consisted of ko-DMEM (Invitrogen), supplemented with 15% ko-SR (Invitrogen), 2 mM GlutaMax (Invitrogen), 1% NEAA (Cambrex Bio Science), 0.1 mM β-mercaptoethanol (Invitrogen), 0.12 ng/mL TGFβ (Sigma–Aldrich, St. Louis, MO), and 4 ng/mL bFGF (R&D Systems).

Chemically defined medium. The CDM culture medium, a modification of the medium used by Vallier *et al.*,[10] consisted of 50% IMDM/50% F12 + Glutamax I (Invitrogen) supplemented with 5 mg/mL HS albumin (HSA; Sigma–Aldrich), 1% chemically defined lipid concentrate (Invitrogen), 450 μM monothioglycerol (Sigma–Aldrich), 7 μg/mL hr-insulin (Invitrogen), 15 μg/mL human holo-transferrin (Sigma–Aldrich), 10 ng/mL bFGF (R&D Systems), and 12 ng/mL Activin A (R&D Systems).

TeSR1 medium and modified TeSR1 medium. The TeSR1 medium was prepared according to the original publication by Ludwig *et al.*[14] with the modification of adding antibiotics. DMEM/F12 (Invitrogen) was supplemented with 2 μg/mL glutathione (Sigma–Aldrich), 45 μg/mL L-ascorbic acid 2-phosphate (Sigma–Aldrich), 10.4 μg/mL transferrin (Sigma–Aldrich), 0.014 mg/L selenium (Sigma–Aldrich), 6 mg/L thiamine (Sigma–Aldrich), 12.9 mg/mL HSA (Sigma–Aldrich), 1:1000 Trace elements B (Cellgro, Herndon, VA), 1:1000 Trace elements C (Cellgro), 22.8 mg/L insulin (Invitrogen), 1 mM Glutamax (Invitrogen), 1% nonessential amino acid solution 100×(Invitrogen), 0.5% penicillin/streptomycin (Cambrex Bio Science), 0.1 mM β-m-EtOH (Cambrex Bio Science), 100 ng/mL bFGF (R&D Systems), 0.6 ng/mL TGFβ (Sigma–Aldrich), 0.127 mg/L pipecolic acid (Sigma–Aldrich), 101 μg/mL gamma amino butyric acid (Sigma–Aldrich), 1:500 chemically defined lipid concentrate 100×(Invitrogen), and 41.54 mg/L LiCl (Sigma–Aldrich).

The mTeSR1 medium containing xeno proteins was purchased from StemCell Technologies (http://www.stemcell.com) and handled according to the manufacturer's instructions.

### *Culture matrices*

Biomaterials. Different biomaterials were tested for undifferentiated hESC culture together with hES medium and hES-CM. Pure Ti metal plates were compared with $TiO_2$-coated Ti plates (Vivoxid, Turku, Finland) and $ZrO_2$-coated Ti plates (Turku Biomaterials Centre, University of Turku, Finland). The coatings were produced using sol-gel dip-coating method. About 10×10 mm pieces were disinfected with 70% ethanol, allowed to air dry, and placed in a four-chamber slide (Nalge Nunc International, Rochester, NY). Chambers without biomaterial were used as a control. The experiment was repeated five times for all three biomaterials. In addition, five different $TiO_2$ coating modifications in respect to sol composition and calcination temperatures were produced on glass slides and tested accordingly (Turku Biomaterials Centre).

The biodegradable tyrosine-derived polymer PDTEC was obtained as sterile plates (Institute of Biomaterials, Tampere University of Technology, Finland) and cut with sterile scissors to fit into culture dishes. The plate pieces were either used as matrix as such or attached to the dish with sterile tissue glue (Tisseel® Duo Quick I + II; Baxter, Deerfield, IL). The poly-L,D-lactide 96/4 (PLDLA) 3D scaffold (Institute of Biomaterials) was disinfected with 70% ethanol, allowed to air dry, and placed on a tissue culture dish. Human ESCs were plated directly on the scaffolds and cultured for up to 7 days.

Purified human ECMs. Human fibronectin coating was tested as a hESC culture matrix with two types of culture media: mhES and CDM. The culture plates were coated with 5 μg/cm² fibronectin from human foreskin fibroblasts (Sigma–Aldrich) at room temperature (RT) for 2 h and tested with the mhES medium. The fibronectin coating was removed after 2 h, and hESCs were plated without washing.[12] Culture plates coated at 4°C overnight with 5 or 20 μg/cm² fibronectin from hFF (Sigma–Aldrich) were tested with the CDM. The fibronectin was removed, and the wells were washed once with phosphate-buffered saline (PBS) (Cambrex Bio Science) before plating the hESCs. BD BioCoat™ Human Fibronectin Cellware 12-well plates (BD Biosciences, Franklin Lakes, NJ) were also tested with both media types by directly plating the hESCs in the respective culture media on the plate brought from 4°C to RT.

A mix of four human ECM components (hECM mix) consisting of 10 μg/cm² collagen type IV from human placenta, 0.2 μg/cm² vitronectin from human plasma, 5 μg/cm² fibronectin, and 5 μg/cm² laminin from human placenta (all from Sigma–Aldrich) was tested in combination with different culture media. Culture plates were coated with a mix of the four human ECM proteins in PBS at least overnight at 4°C or first with collagen IV for 2 h at RT, followed by washing with PBS and overnight incubation at 4°C with the other three components. The coating mix was removed, surfaces were washed once with PBS, and hESCs were plated in appropriate culture medium. The hECM mix was tested with hES, hES-CM, and TeSR1.

Human or bovine serum coatings. HS coating was tested as hESC culture matrix together with hES medium and hES-CM. The culture plates were coated with sterile filtered HS (H1388; Sigma–Aldrich). The culture plates were first incubated with HS for 1 h at RT followed by 1 h drying in the sterile hood.[11] As this coating method was not successful, the coating time was increased to overnight at 37°C in a 5% $CO_2$ incubator. The HS was removed, replaced with culture media, and hESCs plated. The feeder cell–free culture on the HS matrix was performed as described by Stojkovic *et al.*,[11] with a modification of using fresh hES-CM as culture medium instead of conditioned medium recovered from fibroblast-like cells derived from hESCs (hES-dF-CM).

Both 10% HS and 10% FBS coatings were tested together with CDM. Culture plates were coated with 10% heat inactivated HS (type AB; PAA Laboratories GmbH, Pasching, Austria) or 10% heat inactivated FBS (Invitrogen) in IMDM (Invitrogen) and 0.5% penicillin/streptomycin (Cambrex Bio Science). The coated plates were incubated at 37°C, 5% $CO_2$

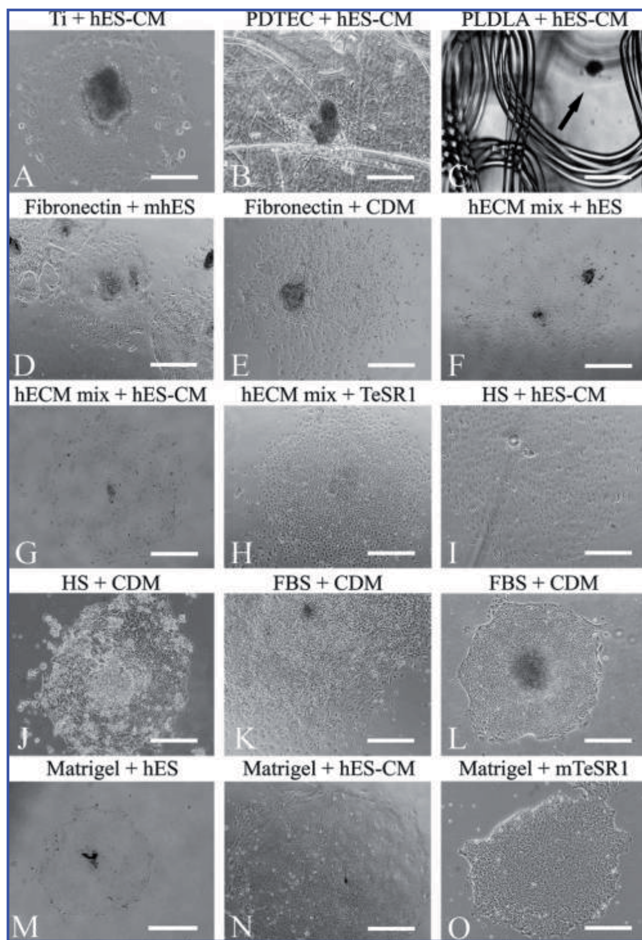


**FIG. 2.** Typical morphology of hESCs on different culture matrices. (**A**) HS293 cultured on Ti in hES-CM for one passage. (**B**) HS293 cultured on PDTEC in hES-CM for one passage. (**C**) Unattached colony of HS237 floating on PLDLA scaffold in hES-CM (arrow). (**D**) HS360 cultured on hFF fibronectin in mhES medium for two passages. (**E**) HS360 cultured on BD BioCoat Human Fibronectin plate in CDM for two passages. (**F**) HS401 cultured on hECM mix in hES medium and (**G**) in hES-CM for two passages. (**H**) HS401 cultured on hECM mix in xeno-free TeSR1 medium for six passages. (**I**) HS401 cultured on HS coating in hES-CM for one passage. (**J**) HS360 cultured on 10% HS coating in CDM for one passage and (**K**) on 10% FBS coating in CDM for five passages. (**L**) HS237 (46X, abnormal X) cultured on 10% FBS coating in CDM for seven passages. (**M**) HS401 cultured on Matrigel in hES medium and (**N**) in hES-CM for two passages. (**O**) HS401 cultured on Matrigel in mTeSR1 medium for 20 passages. Scale bars = 500 μm, except for (**G**) and (**O**) = 200 μm. For abbreviations, see Materials and Methods section. Color images available online at www.liebertonline .com/ten.

incubator from 1 to 7 days and washed once with PBS before plating the hESCs using CDM.

Matrigel. BD Matrigel hESC-qualified Matrix (BD Biosciences) was tested in combination with hES medium, hES-CM, and commercial mTeSR1. Culture plates were coated with Matrigel at 4°C at least overnight, as instructed by the manufacturer. The plate was brought to RT, coating was removed, and hESCs were plated using the appropriate medium.

*Characterization of cells*

Morphologic characterization. The hESC growth on the different matrices was judged primarily by colony attachment and morphology. The growth was monitored daily under a Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V. Amstelveen, The Netherlands). Colonies were judged as undifferentiated if the colony had an even form and structure with defined borders (Fig. 2L, O). The loss of defined borders and the emergence of other cell types were judged as differentiation. The colony differentiation in feeder cell–free hESC culture was typically manifested as the emergence of mesenchymal-like (fibroblast-like) cells, first at the colony borders (Fig. 2K) and then progressively throughout the whole colony (Fig. 2H). This phenomenon is also referred to as autologous feeder formation and leads to loss of hESC colonies in the culture plate. The morphologic characterization of the undifferentiated hESC colonies was confirmed with immunocytochemical staining and fluorescence-activated cell sorter analysis (FACS).

Immunocytochemical staining. The hESCs cultured in feeder cell–free conditions were characterized by immunocytochemical staining with antibodies specific for undifferentiated hESCs (Nanog, OCT-3/4, SSEA-3, SSEA-4, TRA-1-81, and TRA-1-60) and an antibody specific for differentiated hESCs (SSEA-1). The hESC colonies were fixed with 4% paraformaldehyde for 20 min at RT and permeabilized with 0.1% Triton X-100 (Sigma–Aldrich), 1% BSA (Sigma–Aldrich), and 10% normal donkey serum (Sigma–Aldrich) in PBS for 45 min at RT. Following are the primary antibodies that were incubated at 4°C over night: Nanog 1:200 (R&D Systems), Oct-3/4 1:300 (R&D Systems), SSEA-3 1:300 (Novus Biologicals, Littleton, CO), SSEA-4 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA), TRA-1-81 1:200 (Santa Cruz Biotechnology), TRA-1-60 1:200 (Millipore, Billerica, MA), and SSEA-1 1:200 (Santa Cruz Biotechnology). The cells were probed with secondary antibodies for 1 h in the dark at RT. Alexa Fluor 568–conjugated donkey anti-goat IgG, goat anti-mouse IgM, and goat anti-mouse IgG antibodies; Alexa Fluor 488–conjugated donkey anti-goat IgG and goat anti-mouse IgM; and FITC-conjugated anti-Rat IgM antibodies at a dilution of 1:800 were used as secondary antibodies (all from Invitrogen, except anti-Rat IgM, which was from Novus Biologicals). Vectashield mounting medium containing 40,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) was used for nuclei counter staining. The labeled cells were photographed using an Olympus IX51 phase contrast microscope with fluorescence optics and Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan). Human ESC labeled only with secondary antibodies and hFF cells were used as negative controls.

Fluorescence-activated cell sorter analysis. The HS401 hESCs cultured for 28 passages on Matrigel in mTeSR1 medium were analyzed using FACS for SSEA-4 and TRA-1-81 expression. The cells were dissociated with Tryple$^{TM}$ Select (Invitrogen) and counted with a hemocytometer using trypan blue exclusion. For SSEA-4 analysis 0.5% BSA in PBS and for TRA-1-81 analysis 2% FBS in PBS were used as FACS buffers. Cells ($0.1\times10^6$) were probed for 45 min at 4°C with 0.5 μL of phycoerythrin-conjugated anti-human/mouse-SSEA-4 antibody (R&D Systems) or for 30 min at 4°C with

1:200 dilution of TRA-1-81 (Santa Cruz Biotechnology). For TRA-1-81 analysis, the cells were probed with 1:500 dilution of R-phycoerythrin–conjugated anti-mouse IgM secondary antibody (Invitrogen) at 4°C for 20 min. The cells were analyzed using BD FACSAria™ (BD Biosciences). The samples were analyzed in triplicate, and acquisition was set for 20,000 events per sample. R-Phycoerythrin–conjugated goat-anti mouse IgG antibody (Invitrogen) was used as an isotype control, and R-phycoerythrin–conjugated anti-mouse IgM antibody (Invitrogen) as a secondary control. The data were analyzed using FACSDiva Software version 4.1.2 (BD Biosciences, San Jose, CA).

Karyotype analysis. Karyotype analysis was performed on HS401 hESC lines cultured on Matrigel matrix in mTeSR1 medium for 24 passages. The hESCs were transferred back to hFF feeders and cultured using hES medium on hFF feeder cells for 2 to 4 passages before the karyotype analysis. A cytogenetic analysis of 20 metaphase cells was performed using G-banding at Medix Laboratories (Espoo, Finland).

Quantitative RT-PCR. The expression of *Oct-4* was analyzed over time in three of the tested culture systems with quantitative RT-PCR (q-RT-PCR). Total RNA was extracted from HS360 cells cultured on Matrigel in mTeSR1 for 1, 2, and 3 passages and Regea 06/015 cells cultured for 10, 21, and 32 passages. RNA samples were also collected from HS360 cells cultured on hECM mix on TeSR1 for 1, 2, and 3 passages and on 10% FBS coating in CDM for 1 and 2 passages. RNA from three different passages (p83, p84, and p85) of HS360 hESCs cultured on hFF feeder cells in hES medium was collected to serve as a reference level of gene expression. The hESCs were collected from the culture plates, lysed to RLT plus buffer (Qiagen, Valencia, CA), and stored at −70°C until RNA was extracted with Qiagen RNeasy Plus Mini kit according to manufacturer's instructions. The RNA concentration and quality was assessed with NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Fifty nanogram of RNA was transcribed to cDNA in a total volume of 20 μL with Sensicript Reverse Transciription kit (Qiagen) according to manufacturer's instructions for 1 h at 37°C. The cDNA was stored at −20°C, until used in q-RT-PCR analyses. The q-RT-PCR was performed with Applied Biosystems (Foster City, CA) Gene Expression Assays: *POU5F1* (Hs00999632_g1) and *GAPDH* (Hs99999905_m1). *GAPDH*, known to have constant expression in our hESC lines (data not shown), was used as a housekeeping control. The PCR reaction consisted of 3 μL of cDNA in 1:10 dilution, 7.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and 0.75 μL of assay. All samples, $dH_2O$ controls from cDNA synthesis, and no template controls were analyzed as three replicates. The q-RT-PCR was carried out with Applied Biosystems 7300 Real-time PCR system: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 0.15 min at 95°C and 1 min at 60°C. The data were analyzed with 7300 System SDS Software (Applied Biosystems).

$C_\tau$ values were determined for every reaction and qualified for analysis if the standard deviation of the three replicate values was <0.5. Relative quantification was calculated with the $2^{-\Delta\Delta C_\tau}$ method.[33] The data were normalized with the expression of the housekeeping gene *GAPDH*, and the expression level of *Oct-4* in HS360 hESCs cultured in standard culture conditions on hFF feeder cells in hES medium was used as reference level. The data are presented as mean fold change values as compared to the reference level. Standard deviation is presented as error bars. For determining statistical significance, the Mann–Whitney *U*-test for unmatched pairs was used. $p$-Value <0.05 was considered statistically significant.

## Results

The maximum passages for which each culture matrix and media combination supported hESC culture are summarized in Table 1.

### *hESC culture on selected biomaterials*

Ti, $TiO_2$, $ZrO_2$, PDTEC plate, and PLDLA scaffold were tested as hESC attachment and culture matrices together with hES and hES-CM media. The hESCs did not attach to any of the biomaterials in the presence of unconditioned hES medium, but some attachment occurred on Ti, $TiO_2$, $ZrO_2$, and PDTEC when hES-CM was used (Fig. 2A, B). The hESCs did not attach to the PLDLA scaffold (Fig. 2C) or to the uncoated chamber glass slide used as a control material even with hES-CM. The hESC colonies were very fragile and easily detached from the biomaterials. The $TiO_2$ was also tested as five modifications with similar results (data not shown). The hFF cells routinely used as feeder cells, however, attached to and grew on PDTEC and on all of the Ti materials (data not shown).

### *hESC culture on purified human ECMs*

Human fibronectin was tested as a culture matrix for undifferentiated hESCs according to the method described by Amit and Itskovitz-Eldor[12] using mhES media as well as the CDM used for hESC propagation by Vallier *et al*.[10] Human FF fibronectin and BD BioCoat Human Fibronectin Cellware were tested. Neither type of fibronectin coating supported undifferentiated hESC culture beyond the second passage (i.e., 8 days) with either of the media types tested (Fig. 2D, E and Fig. 3A, B). The hESCs quickly underwent differentiation and attached poorly after passaging. Increasing the concentration of hFF fibronectin to 20 μg/cm$^2$ did not yield any better results.

The hECM mix was tested together with hES medium, hES-CM, and defined xeno-free TeSR1 medium. Human ESC colonies attached to the hECM mix in all media types tested. In hES and hES-CM media, the hESC colonies were thin, fragile, and quickly differentiated toward a mesenchymal-like phenotype within two passages (8 days) (Fig. 2F, G). In TeSR1 medium, the hESCs underwent progressive differentiation and poorer attachment in subsequent passages (Fig. 2H). The hESCs were cultured for maximum of 7 passages under these conditions, after which all cells had a differentiated morphology and lost the expression of OCT-3/4, a marker of undifferentiated hESCs (Fig. 3C, D).

### *hESC culture on serum coatings*

On HS coating the hESC colony pieces either did not attach at all or grew poorly and easily detached during culture

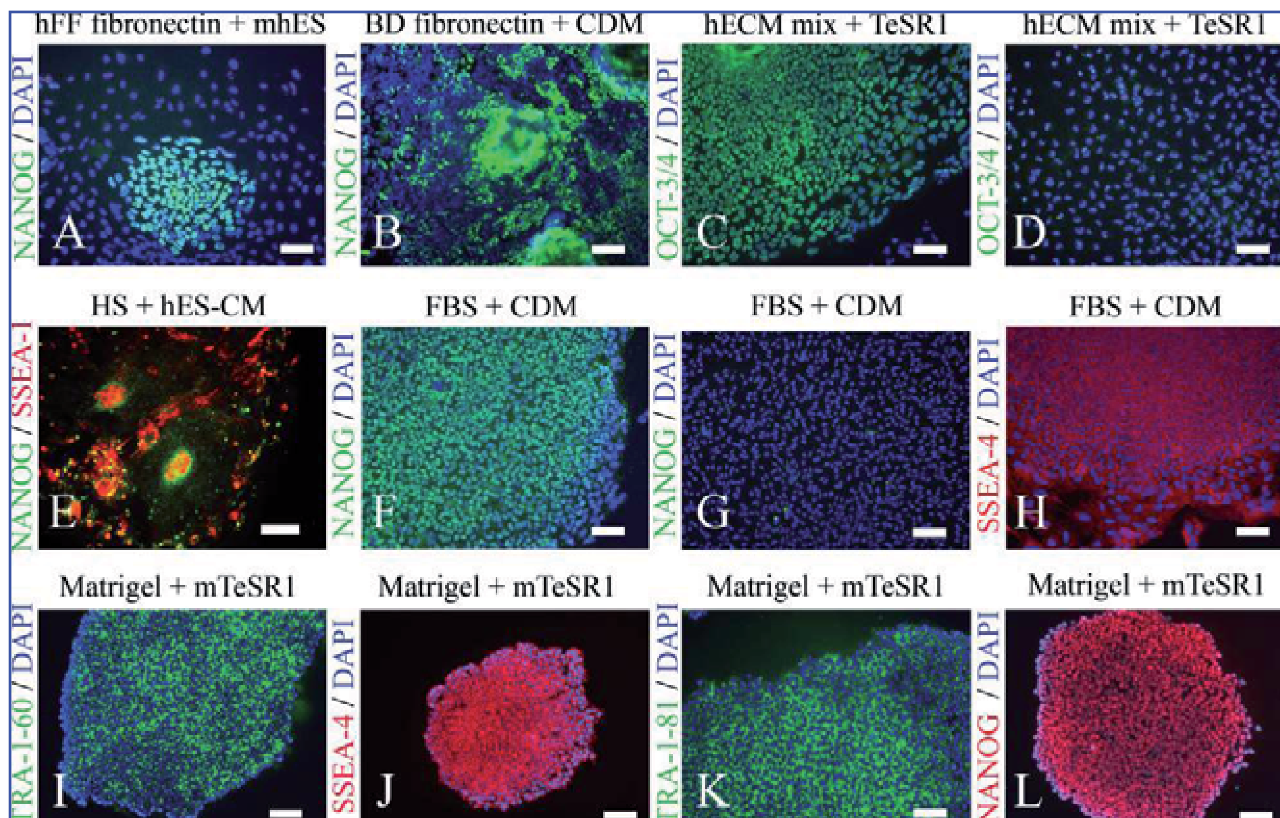


**FIG. 3.** Human ESC colonies immunostained with markers of undifferentiated hESCs (Nanog, OCT-3/4, SSEA-4, TRA-1-81, and TRA-1-60) and a marker for differentiated hESCs (SSEA-1). (**A**) HS360 cultured on fibronectin derived from human foreskin fibroblasts in mhES medium for two passages. Note the autologous feeders around the colony that have lost the expression of Nanog. (**B**) HS360 cultured on B&D BioCoat fibronectin plate in CDM for one passage showing ragged colony morphology and only moderate Nanog expression. (**C**) HS360 cultured on hECM mix in TeSR1 medium for two passages showing strong expression of OCT-3/4 and (**D**) HS401 for seven passages showing the loss of colony morphology and OCT-3/4 expression. (**E**) HS237 cultured on HS coating in hES-CM for two passages showing expression of both Nanog and SSEA-1 in a double staining. (**F**) HS360 cultured on 10% FBS coating in CDM for five passages showing strong expression of Nanog. (**G**) HS360 cultured on 10% FBS coating in CDM for 10 passages have lost the expression of Nanog. (**H**) HS237 (46X, abnormal X) cultured on 10% FBS coating in CDM for 13 passages showing strong expression of SSEA-4. (**I–L**) HS401 cultured on Matrigel in mTeSR1 for 23 passages showing strong expression markers of undifferentiated hESCs. Scale bars = 200 μm.

with hES medium. In hES-CM medium, the colonies attached more readily compared to hES medium, and the observed colony growth and morphology was generally better in the first passage. Even in hES-CM medium, the hESC colonies underwent excessive differentiation (Fig. 2I) and could only be cultured for the maximum of three passages, after which the colonies had completely differentiated and expressed SSEA-1, a marker of differentiated hESCs, with only moderate expression of Nanog, a marker of undifferentiated hESCs (Fig. 3E). No difference in attachment or hESC growth was observed when 1% insulin, transferrin, and selenium supplement was added to the hES-CM medium (data not shown), as described by Stojkovic *et al.*[11]

We also tested culture dishes coated with cell culture medium containing 10% HS or 10% FBS together with CDM. Again, neither of the matrices supported long-term undifferentiated hESC culture together with the CDM. On 10% HS coating, the hESCs (Fig. 2J) could only be cultured for up to two passages in CDM, after which the cells either differentiated or detached and were lost. On 10% FBS coating, the hESCs progressively differentiated (Fig. 2K), and after 10 passages, autologous feeder cells had taken over the entire surface of the culture dish and hESCs had lost their expression of Nanog (Fig. 3F, G). The abnormal HS237 hESC line (46X, abnormal X) was cultured for 13 passages on 10% FBS coating with comprehensive attachment and even, round colonies with defined borders (Fig. 2L). The hESCs showed no differentiation toward other cell types after the first passages and strongly expressed the surface markers of undifferentiated hESCs (Fig. 3H). The HS237 (46X, abnormal X) performing ideally on 10% FBS coating was transferred to 10% HS coating at the ninth passage and cultured for five more passages on this substratum, again with ideal behavior. After a total of 14 passages, the culture was aborted.

### *hESC culture on Matrigel*

The combination of Matrigel and hES medium could not support undifferentiated hESC culture beyond the second

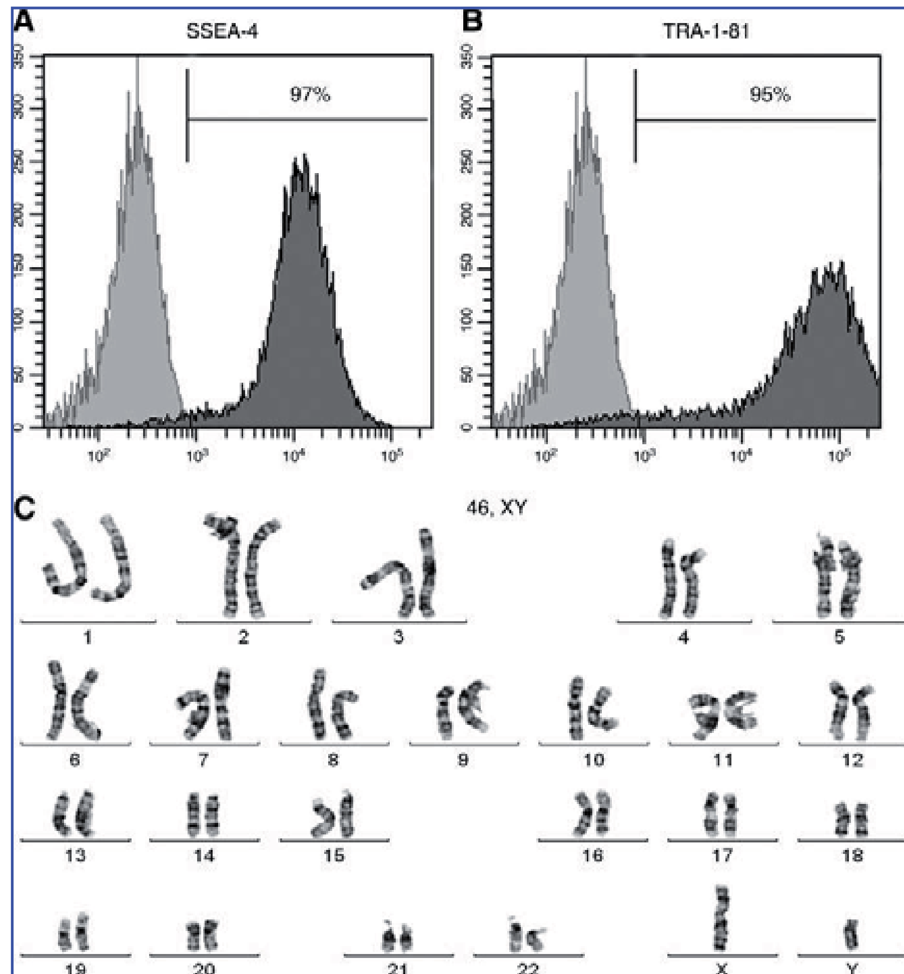


**FIG. 4.** Characterization of HS401 hESCs cultured on Matrigel in mTeSR1 medium. FACS analysis confirmed that (**A**) 97% of hESCs were SSEA-4 positive and (**B**) 95% were TRA-1-81 positive after 28 passages. (**C**) The karyotype of the cell line was confirmed to be normal diploid (46, XY) after 24 passages in the culture system.

passage. With hES-CM the hESCs could be cultured for five passages, after which the colonies were completely lost due to inadequate attachment or because there were only one or two partially undifferentiated colonies left in the well and no further passaging was feasible. Compared to culture on hECM mix, the colonies were, however, much thicker and more solid on Matrigel (Fig. 2G, N).

With commercial mTeSR1 medium the hESCs underwent differentiation in the beginning of the culture, but after a few passages of selection most colonies had an undifferentiated morphology. The HS401 hESCs were successfully cultured over 30 passages with an undifferentiated morphology (Fig. 2O). The colonies attached properly and showed little differentiation and strong expression of markers of undifferentiated hESCs in both immunocytochemical stainings (Fig. 3I–L), and as confirmed with FACS analysis. According to FACS analysis, 97% of HS401 hESCs were SSEA-4 positive and 95% were TRA-1-81 positive after 28 passages on Matrigel in mTeSR1 (Fig. 4A, B). The karyotype of the cell line was confirmed to be normal diploid (46, XY) after 24 passages in the culture system (Fig. 4C). The Regea 06/015 hESC line has to date also been cultured using Matrigel and mTeSR1 over 30 passages with similar undifferentiated morphology and protein expression in immunocytochemical stainings and FACS as well as normal karyotype after long-term feeder-free culture (data not shown).

### *Expression of Oct-4 in hESCs cultured with different feeder cell–free culture systems*

The relative gene expression of Oct-4 was studied in hESCs cultured with three matrix–media combinations: Matrigel combined to mTeSR1 medium that supported long-term undifferentiated hESC culture and two non-supportive culture methods based on the hECM mix together with TeSR1 and 10% FBS coating together with CDM. The constant expression of Oct-4 in hESCs cultured in the standard conditions on hFF was used as a reference level. The lower expression of Oct-4 in reference sample as compared to the feeder cell–free samples is explained by the feeder cell RNA present in the reference sample. The expression of Oct-4 remained constant throughout the long-term culture on Matrigel between the samples of the two hESC lines from passages 1, 2, 3, 10, 21, and 32 (Fig. 5). As anticipated, on hECM in TeSR1 medium and on FBS coating in CDM, the Oct-4 expression level decreased as colonies differentiated and detached from early on. On hECM mix in TeSR1 medium the expression of Oct-4 decreased significantly ($p < 0.05$) at passages 2 and 3 compared to culture on Matrigel. Also on 10% FBS coating, the expression level decreased from first passage to the second although the difference was not statistically significant.

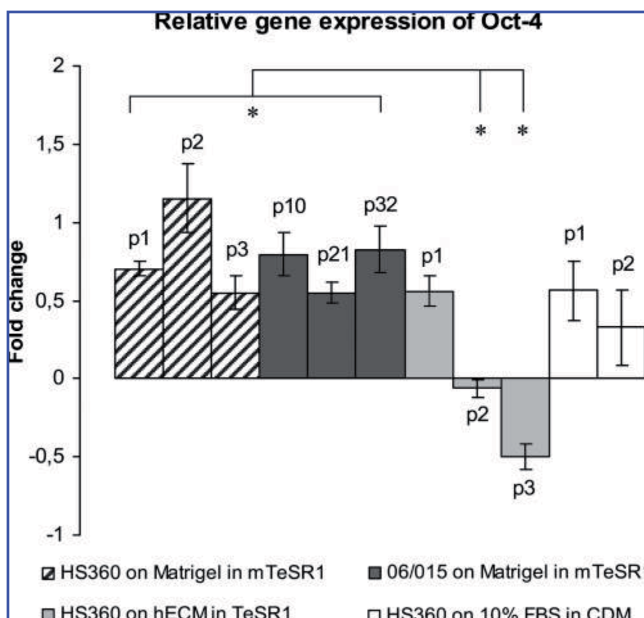


**FIG. 5.** Relative gene expression of Oct-4 in hESCs at different passages cultured with three different matrix–media combinations: on Matrigel in mTeSR1, on hECM mix in TeSR1, and on 10% FBS in CDM. The expression level of Oct-4 in HS360 hESCs cultured on hFF with hES medium was set as a reference baseline ($*p < 0.05$).

### Discussion

Eliminating the feeder cells from the hESC culture system would substantially reduce the cost and labor of hESC culture, offer more defined culture systems that could be more easily reproduced, and enable the scale-up of hESC production for potential clinical use. The human ECM proteins, synthetic biomaterials, or their combinations would offer a xeno-free alternative to feeder cells that could be validated to correspond to GMP-quality requirements.

Feeder cell–free hESC culture is extremely demanding. The maintenance of hESC pluripotency is interplay between the matrix and the soluble factors provided by the culture medium. Especially in the absence of CM, the feeder cell–free culture conditions described so far rely on high concentrations of growth factors, which reflects the currently insufficient knowledge about the maintenance of self-renewal and pluripotency of hESCs. The published culture methods have also been difficult to reproduce in different laboratories with different cell lines. It has been speculated that the origin of the hESC line as well as the derivation and culture conditions, media, matrix, and passage numbers used in different studies have a major effect on the results of feeder cell–free and other experiments conducted with hESC lines. The hESC lines are also genetically different, and it is possible that some cell lines are better suited for special purposes and more prone to grow on anything than other lines.[34,35] This makes undefined culture systems highly undesirable, especially if different hESC lines show different responses to the culture conditions.

To date, biomaterials as culture matrices have not been adequately tested for undifferentiated propagation of hESC culture. The hESCs in general do not easily attach to standard cell culture plastics; if they do attach, the colonies undergo spontaneous differentiation. The biomaterials tested here did not support hESC attachment or growth as such, even though they were nontoxic to cells because the hFF cells were able to attach to and grow on them.

The hESC attachment was clearly better on the hECM proteins tested compared to the synthetic biomaterials. This strongly emphasizes the role of ECM in both attachment and hESC self-renewal. Also, the importance of the soluble factors secreted by the fibroblast feeder cells for the attachment and growth of hESCs was evident as the hES medium was inferior to hES-CM in combination with both the biomaterials and hECM proteins tested. Nevertheless, the hES-CM collected from hFF cells did not support undifferentiated hESC culture on any of the matrices tested, in contrast to MEF-CM, that is widely used.[8,36,37] Other CM types successfully used in hESC culture are usually collected from fibroblasts of fetal origin or from fibroblast-like cells derived from hESCs.[11,38,39] In our experiments, the hES-CM was collected from confluent monolayers that at the same density support undifferentiated hESC culture as feeder layers. This further emphasizes the role of the cell–ECM protein and cell–cell interactions in the maintenance of hESC pluripotency.

The hECM mix tested has previously been reported to support undifferentiated hESC culture by Ludwig and coworkers together with xeno-free TeSR1 medium.[14] In our own experiments, different human ECM proteins were initially tested individually as culture matrices using hES medium and hES-CM. The purified proteins alone were all inferior compared to the mix of all four (data not shown), consistent with the results of Ludwig *et al*. We previously reported that the hECM mix together with the xeno-free TeSR1 medium was not sufficient for maintaining undifferentiated hESC culture.[40] Here, we even tested the medium composition as described in the original publication relating to the concentrations of HSA, insulin, selenium, and transferrin. The human ECM mix and xeno-free TeSR1 medium did not support undifferentiated hESC culture of our cell lines beyond the early passages but instead led to detachment and loss of pluripotency markers. The decrease of Oct-4 expression already in the early culture passages was consistent with the morphological findings. The expression level of Oct-3/4 has been shown to be a sensitive indicator of pluripotency status in ES cells and changes even less than twofold to be biologically relevant.[41]

Human and animal sera are rich in ECM proteins and were therefore tested as attachment bases in feeder cell–free hESC culture. The attachment of hESC colony pieces to HS-coated culture dishes was greatly improved by a longer incubation time at higher temperature, which allowed enough ECM proteins to attach to the culture dish surface to allow hESC attachment and growth. HS has been used as a hESC culture matrix by Stojkovic *et al*.[11] The same HS was used for the coating in this study. Stojkovic *et al*.[11] used a different hESC line (H1) that was derived and propagated under conditions (e.g., MEF feeders) that differed dramatically from those used for the hESC lines in the present study. In addition, the CM they used differed from the one used here, and these factors may underlie their success in culturing hESCs on HS coating. The FBS coating successfully used by Vallier *et al*.[10] was clearly better than HS as a hESC culture

matrix. The addition of serum is essential to most cell culture protocols. FBS is generally more suitable for cell culture, and it is likely that HS both lacks the components that are beneficial and contains components that are harmful to cells. Still, even FBS was an inadequate substratum for long-term hESC culture with CDM and hESC colonies differentiated or were lost within the first culture passages. Consistently, the expression level of Oct-4 decreased within the second passage and would most likely have continued to decrease in subsequent passages if further passaging would have been feasible. The successful culture of the karyotypically abnormal HS237 cells on FBS coating with CDM indicates that the karyotypic abnormality had a positive effect in that culture system. The FBS coating and CDM did not support undifferentiated culture of either of the karyotypically normal hESC lines HS401 or HS360. Feeder cell–free culture conditions favor the occurrence of karyotypic abnormalities,[14,31] and even though the karyotypic abnormality of the HS237 cell line was gained under standard culture conditions, our results indicate that karyotypically abnormal hESC lines are easier to culture under feeder cell–free culture conditions.

All of the biomaterials and human ECM proteins as well as sera coatings tested were inferior to the undefined, xeno-product Matrigel. The attachment and morphology of the hESC colonies was better on Matrigel compared to the hECM mix tested in parallel with hES and hES-CM media. The combination of Matrigel and mTeSR1 medium successfully supported long-term undifferentiated hESC culture of two hESC lines with appropriate colony morphology and marker expression. The culture on Matrigel in mTeSR1 also showed constant Oct-4 expression levels between different passages. Unfortunately, both the Matrigel matrix and the mTeSR1 medium are far from xeno-free, and thus this culture system is unsuitable for use in the derivation and culture of clinical-grade hESCs. Our systematic testing of different culture matrices and media combinations for long-term undifferentiated hESC culture showed that feeder cell–free hESC culture is extremely difficult and that no globally reproducible, cost-effective, xeno-free, and feeder cell–free hESC culture method exists. There is a growing need for development of such culture conditions for hESCs as the phase of clinical trials of hESC-based cellular therapies is fast approaching.

## Acknowledgments

We would like to thank the entire staff of Regea and especially Professor Outi Hovatta for providing the hESC lines used in this study. We would also like to thank Vivoxid and Mika Pelto (Tampere University of Technology) for providing us with biomaterials. This work was supported by the TEKES, the Finnish Funding Agency for Technology and Innovation, the Academy of Finland, the Competitive Research Funding of the Pirkanmaa Hospital District, and the University of Tampere.

## Disclosure Statement

No competing financial interests exist.

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*Received: June 3, 2008*
*Accepted: November 7, 2008*
*Online Publication Date: January 8, 2009*

# Accepted Manuscript

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PII: S1873-5061(11)00111-5
DOI: doi: 10.1016/j.scr.2011.08.005
Reference: SCR 176

To appear in: *Stem Cell Research*

Received date: 7 January 2011
Revised date: 19 August 2011
Accepted date: 22 August 2011

Please cite this article as: Hongisto, Heidi, Vuoristo, Sanna, Mikhailova, Alexandra, Suuronen, Riitta, Virtanen, Ismo, Otonkoski, Timo, Skottman, Heli, Laminin-511 expression is associated with the functionality of feeder cells in human embryonic stem cell culture, *Stem Cell Research* (2011), doi: 10.1016/j.scr.2011.08.005

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**Laminin-511 expression is associated with the functionality of feeder cells in human embryonic stem cell culture**

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**Abstract**

Fibroblast feeder cells play an important role in supporting the derivation and long term culture of undifferentiated, pluripotent human embryonic stem cells (hESC). The feeder cells secrete various growth factors and extracellular matrix (ECM) proteins into extracellular milieu. However, the roles of the feeder cell-secreted factors are largely unclear. Animal feeder cells and use of animal serum also make current feeder cell culture conditions unsuitable for derivation of clinical grade hESCs. We established xeno-free feeder cell lines using human serum (HS) and studied their function in hESC culture. While human foreskin fibroblast (hFF) feeder cells were clearly hESC supportive, none of the established xeno-free human dermal fibroblast (hDF) feeder cells were able to maintain undifferentiated hESC growth. The two fibroblast types were compared for their ECM protein synthesis, integrin receptor expression profiles and key growth factor secretion. We show that hESC supportive feeder cells produce laminin-511 and express laminin-binding integrins α3ß1, α6ß1 and α7ß1. These results indicate specific laminin isoforms and integrins in maintenance of hESC pluripotency in feeder-dependent cultures. In addition, several genes with a known or possible role for hESC pluripotency were differentially expressed in distinct feeder cells.

Highlights

- Human foreskin feeders are clearly hESC-supportive in contrast to dermal cells.
- Several differences in hESC-supportive and non-supportive feeder cells were found.
- hESC-supportive feeders produce laminin-511 and express laminin binding integrins.
- Other possible hESC pluripotency regulators were also differentially expressed.

Abbreviations: hESC, human embryonic stem cell; ECM, extracellular matrix; HS, human serum; hFF, human foreskin fibroblast; hDF, human dermal fibroblast; mEF, mouse embryonic fibroblast; FBS, fetal bovine serum; GMP, good manufacturing practice; CM, conditioned medium; hiPSC, human induced pluripotent stem cell; FGF, fibroblast growth factor; TGFß, transforming growth factor beta; ActA, activin A; KAL1, Kallmann syndrome 1 sequence; mESC, mouse embryonic stem cell.

Key words: Human embryonic stem cell, Feeder cell, Xeno-free culture, Extracellular matrix, Laminin, Integrin

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**1. Introduction**

Undifferentiated human embryonic stem cells (hESC) are traditionally cultured on mouse embryonic fibroblast (mEF) feeder cells in either fetal bovine serum (FBS) or serum replacement supplemented culture medium[1, 2]. The exposure of hESCs to reagents of animal origin is likely to contaminate the cells with animal pathogens and non-human, immunogenic molecules [3, 4]. Future clinical use of stem cell based products requires defined, Good Manufacturing Practice (GMP) compatible culture systems that are free of animal-derived reagents.

There have been two main approaches for replacing mEFs: the use of human feeder cells and the development of feeder-independent culture conditions. The feeder-independent culture systems often rely on the use of Matrigel extracellular matrix (ECM) preparation and mEF conditioned hESC medium (CM) [5]. More defined culture methods based on human basement membrane protein coatings and serum-free culture media have been described [6]. Many of the feeder-independent culture conditions, however, are not defined and have been difficult to reproduce with different hESC lines [7, 8]. The derivation of new hESC and human induced pluripotent stem cell (hiPSC) lines is still routinely performed on supportive feeder layers, although there are a few recent studies describing hESC and hiPSC lines established on ECM preparations [6, 9-12].

Feeder cells play a crucial role in maintaining undifferentiated hESC morphology and pluripotent status in feeder-dependent culture conditions. Human fibroblasts originating from different sources show varying capacity in supporting undifferentiated hESC culture. Clearly, the most supportive fibroblasts are derived from fetal tissue [13-17]. Ethical considerations in using aborted human fetuses make this tissue source an unattractive option. We have derived and successfully propagated eight Regea hESC lines using commercial human foreskin fibroblast (hFF) (CRL-2429, ATCC) feeder cells [18, 19]. The hFFs have become the most commonly used human feeder cell type for hESC derivation and culture [20-23], and even human dermal fibroblasts (hDF) have been used for hESC propagation [13, 24]. Skin is an easily accessible tissue source for derivation of fibroblasts, but there are notable differences between the capacities of different fibroblast lines to support hESC cultures [25]. Reasons for this are poorly understood.

The fibroblast feeder cells support hESCs by secreting growth factors and ECM components to the culture medium and also by directly interacting with the hESCs through cell-cell contacts. The factors secreted by fibroblast feeder cells have been studied by analyzing fibroblast CM [26-30] and de-cellularized matrices with mass spectrometry [31]. Several ECM proteins such as collagens,

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fibronectin, laminins, nidogen and heparan sulfate proteoglycans have been suggested as key factors provided by the hESC-supportive feeder cells [26, 27, 30, 31]. Vitronectin [32, 33], fibronectin [34], laminin [5, 35] and a combination of collagen IV, vitronectin, fibronectin, and laminin [6], have been used as substrata in feeder-independent culture of hESCs, showing that these ECM proteins, in combinations with specific media and growth factors, support attachment and proliferation of hESCs.

In addition to ECM proteins, growth factors provided by the feeder cells or in the culture medium are essential for hESC growth. The central role of basic fibroblast growth factor (bFGF) in hESC self-renewal is well established [36, 37]. Basic FGF and transforming growth factor beta (TGFß) family members TGFß, activin A (ActA) and nodal [38, 39] co-operate to maintain undifferentiated hESC growth. The gene expression and secretion of these key growth factors have been shown to differ between hESC-supportive and non-supportive feeder cells [25, 40].

In this study, we have compared the hESC-supporting capacity of in-house derived, hDF feeder cells to the commercial hFF feeder cells in a xeno-free culture system, based on the use of HS-containing culture medium. We further studied the differences in ECM gene expression and synthesis and secretion of key growth factors between these fibroblasts. We found significant differences in the capacity of the different fibroblast cell lines to support hESC self-renewal and in the production of distinct candidate proteins such as laminin-511.

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## 2. Results

### 2.1 Derivation and culture of xeno-free human dermal fibroblasts

Our initial goal was to derive xeno-free hDF feeder cells that could be used for the production of GMP-grade, clinical quality hESCs. A total of 6 pediatric skin tissue pieces were donated in context of surgery and 5 cell lines were successfully derived from the tissues. The methods were extensively optimized to find simple, effective and eventually GMP- compatible derivation, culture and cryo-banking procedures. Explant culture was found superior to enzymatic digestion of dermis with collagenase, due to simplicity and low cost. HS concentrations from 5% to 20% were tested and 15% HS was found adequate for optimal growth. Heat inactivation (30 min at 56ºC) of HS substantially decreased growth rate and quality of the culture. The outgrowth of fibroblasts was commonly detected within a week. The fibroblast growth typically slowed down after 6-7 passages. All five hDF lines were successfully recovered after cryopreservation.

When used as hESC feeder cells, none of the five hDF lines could support the undifferentiated state of hESCs. Our routinely used, supportive hFF CRL-2429 line was transferred to identical HS-based culture system for comparison. On hFF CRL-2429 the hESCs retained their undifferentiated morphology (data not shown) confirming that hESC differentiation was not caused by the xeno-free feeder cell culture conditions, but originated from differences between the fibroblast cell lines. To further study the factors behind the difference in hESC supportiveness, we chose the hDF 001/08 line for further functional studies and compared its performance and characteristics to the hFF CRL-2429 feeder cells. The hESC supporting capacity of the fibroblasts was first analyzed in depth with two hESC lines and further the ECM expression profile and key growth factor secretion of both feeder cell types were studied. An overview of the workflow is presented in figure 1.

### 2.2 hFFs provide stable support for undifferentiated hESC growth in contrast to hDFs

Two independently-derived hESC lines, Regea 07/046 and Regea 08/017, were cultured on both hFF CRL-2429 and hDF 001/08 fibroblast feeder cells. The cultures were analyzed in depth in terms of hESC colony morphology, proliferation and expression of pluripotency markers on both RNA and protein level. The hESC colony morphology was markedly different throughout the culture on the two types of feeder cells. On the hFF cells, hESC colonies were flat and smooth with well-defined edges (Fig. 2A). The morphology remained unchanged through 10 passages, corresponding to 2.5 months of culture and the hESCs were immunoreactive for Nanog antibody. On hDF 001/08 feeder cells, hESCs underwent progressive differentiation with corresponding morphological changes manifested as loss of smooth structure and defined colony edges (Fig. 2A),

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and Regea 08/017 and Regea 07/046 hESC lines could be cultured for a maximum of 8 and 6 passages, respectively, on these feeders before all colonies had differentiated. The proliferation rate of the hESCs, as measured by bromodeoxyuridine (BrdU) incorporation assay at passage 1-3, was slightly higher on hFFs compared to hDF feeders (Fig. 2B).

Differentiation status of the hESCs on both feeder cell types was analyzed with relative quantitative RT-PCR (q-PCR) analysis of four pluripotency markers: Nanog, POU5F1, TDGF, and DNMT3B. In accordance with the morphological changes, q-PCR analysis showed progressive loss of all four markers for the hESCs cultured on hDFs compared to hESCs cultured on hFFs over six passages (Fig. 2C). This was further confirmed at protein level by flow cytometry analysis of TRA-1-81 and OCT-3/4 expression. Both hESC lines gave similar results, shown for Regea 07/046 (Fig. 2D). Taken together, the analysis confirmed that the hFFs provided a stable, xeno-free feeder cell line to support long-term culture of undifferentiated hESCs, whereas hDF feeder cells did not support hESC self renewal.

**2.3 hDFs show significant down-regulation of several laminin and integrin subunits compared to hFFs**

In order to understand the functional differences between the two feeder cell lines in hESC supportiveness, the hFF CRL-2429 and hDF 001/08 feeder cells were compared for the expression of 84 ECM and ECM-related protein-coding genes by q-PCR array analysis. The hDFs were compared to hFF fibroblast feeder cells for the expression of each gene. Detailed results of this experiment can be found in Supplementary Table 1. Eighteen genes were down-regulated at least two fold and 12 genes up-regulated at least two fold in the hDFs compared to the hFF cells (Fig. 3A). Laminin subunits α1, α3, ß3 and integrin subunits α3, α6, α7 were among the down-regulated genes. The expression of additional laminin chains α4, α5 γ2, and B-CAM in both feeder cell types was studied by q-PCR. Altogether, the laminin subunits α1, α3, α5 and ß3 were significantly (over two fold) down-regulated in hDFs, indicating lower expression of laminins -111, -332 and -511. Accordingly, the expression levels of integrin subunits α3, α6, α7 forming laminin binging integrins α3ß1, α6ß1, α6ß4, α7ß1 were also significantly lower in hDF cells. (Fig. 3B and Supplementary Table 2). Other genes of interest were for example E-cadherin type I and vitronectin that were down-regulated 2.5 and 2.7-fold, respectively in the hDF cells. The largest difference in expression was for Kallmann syndrome 1 sequence (KAL1), 26-fold lower in hDF than hFF cells (Supplementary Table 1).

**2.4 Laminin-511 is synthesized by the hFF and mEF but not by the hDF cells**

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The production of laminin α5 chain was studied at protein level. Human ESC-supportive hFF CRL-2429 cells, mEF cells, as well as four non-hESC supportive hDF cell lines (hDF 002/06, hDF 003/06, hDF 001/07 and hDF 001/08) were labelled over night by $^{35}$S-methionine, followed by immunoprecipitation of the laminin α5 chain from the culture supernatant. Importantly, laminin α5 chain was synthesized only by the hESC-supportive hFF and mEF cells but not by any of the hDF cells (Fig. 4A). In accordance with the immunoprecipitation data, indirect immunofluorescence labelling for laminin α5 showed that the hFF express laminin α5, even if not abundantly, where as there was no expression by any of the hDF lines (Fig 4B).

**2.5 hDFs secrete less TGFß compared to hFFs**

The hFF CRL-2429 and hDF 001/08 feeder cells were compared for their secretion of key growth factors bFGF, TGFß and ActA, known to be important for hESC maintenance. Human ESC culture medium without added exogenous bFGF was incubated overnight on cultures of both feeder cell types and analyzed for the secretion of growth factors by enzyme-linked immunosorbent assay (ELISA). Both fibroblast types secreted undetectable amounts of bFGF (data not shown). They also secreted equal amounts of ActA. After irradiation, the secretion of ActA increased more than two fold for both feeder cell types (Fig. 5A). In contrast, the hFF cells secreted 0.6 ng/ml more TGFß than hDF cells (mean secretion 856 vs. 304 pg/ml; $p<0.01$) (Fig. 5B). To test whether the difference in TGFß production by the feeder cells affected hESC phenotype, the hESCs were cultured on hDF 001/08 feeder cells in standard hESC culture medium containing 8 ng/ml bFGF, supplemented with recombinant TGFß. The addition of 0.6 ng/ml TGFß did not have a relevant impact on hESC colony morphology or to percent of TRA-1-81 positive cells in flow cytometry analysis over 6 passage culture period (Fig. 5 C and D). On the contrary, a higher concentration of 6.0 ng/ml TGFß rescued the hESC colony morphology to some extent (Fig. 5C) and increased the TRA-1-81 positivity of the cells from an average of 51% to 82% during the short term culture (Fig. 5D).

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**3. Discussion**

Feeder cells contribute to maintenance of hESC morphology and pluripotent status in feeder-dependent culture conditions by mechanisms that are poorly known. Likely the feeder cells will be replaced with more defined, feeder-independent hESC culture conditions in the future. Many serum-free media formulations for feeder-independent hESC culture have been published and some, such as mTeSR1 (Stemcell Technologies) [41] and STEMPRO hESC SFM (Life Technologies, Invitrogen) are commercially available. These media are routinely used in combination with xeno-derived, undefined matrices like Matrigel or Geltrex but have been shown to support pluripotent stem cell culture also with defined substrates like vitronectin [32] and laminin-511 [35]. Although the long-term culture of undifferentiated pluripotent stem cells is possible in these feeder-independent conditions, the media still contain undefined and xeno-derived compounds and the success of deriving new pluripotent stem cell lines, both hESC and hiPSC, in completely defined, xeno- and feeder-free conditions is low. Defining the factors provided by fibroblast feeder cells and revealing their complex mechanisms in hESC maintenance provides valuable tools for further development of feeder-independent derivation and culture methods.

We studied the differences in ECM and growth factor production between hESC-supportive and non-supportive human fibroblast feeder cells. Many of the genes down-regulated in the non-hESC supportive hDF feeder cells were distinct laminin isoforms and laminin related integrin subunits that have been reported to be associated with undifferentiated hESCs. Laminins are trimeric glycoproteins consisting of one α (1-5), one ß (1-3), and one γ (1-3) chain [42], and are the first ECM proteins synthesized by the mouse blastocyst [43]. A number of studies have shown that in addition to embryogenesis, the specific laminin isoforms regulate cell adhesion, proliferation, migration and differentiation [42, 44]. We have previously shown that undifferentiated hESCs synthesize and deposit laminin-511 (α5ß1γ1) and -111 (α1ß1γ1) and that laminin-511, purified from human cells, can be used as a supportive matrix in feeder-independent hESC cultures [45]. These results have been confirmed by others for both hESCs [35, 46, 47] and mESCs [48], indicating that laminin-511 has a central role in the maintenance of hESC survival and pluripotency. Some studies have also reported laminin-332 (α3ß3γ2) to function as hESC substrata [46]. In the present study, laminin-511 was found to be produced by the most commonly used hESC-supportive feeder types, hFFs and mEFs, but not by the non-supportive hDF feeder cell lines. This indicates that laminin-511 might also act in hESC self-renewal through feeder cells, yet the possible mechanism remains to be studied.

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The cell interactions to laminins as well as other ECM proteins are mediated mainly through integrins, heterodimeric cell membrane receptors of one α (1-18) and one ß (1-8) subunit [49]. Human ESCs synthesize several integrin subunits: at leats α3, α5, α6, α7, αV, ß1, ß5 and interaction with the hESC-critical laminins is mediated through integrins α6ß1 and α3ß1 and Lutheran/B-CAM receptor, an Ig-superfamily member [5, 35, 45-47]. In our study, the integrin subunits α3, α6 and α7 forming the laminin-binging integrins α3ß1, α6ß1, α6ß4, α7ß1, were down-regulated in the hDFs as compared to hFF cells. Given that hESCs, as well as hFFs, produce laminins-111 and -511, we suggest that the hFF feeder cells support hESCs, at least in part, due to ideal laminin synthesis and the presence of the appropriate set of laminin-binding receptors. Taken together, our results suggest that the interplay between hFFs and hESCs likely depends on crucial signals originating from the ECM environment provided by the feeders.

In addition to genes coding for laminins and integrins, other interesting genes showed lower expression in non-hESC-supportive hDF feeder cells. One of them was the gene coding for vitronectin. Vitronectin has been suggested as one of the defined matrix alternatives to Matrigel in feeder-independent hESC cultures [32]. Accordingly, a recombinant, functional N-terminal domain of vitronectin has been shown to enable long-term feeder-independent hESC cultures [50]. Vitronectin binds to insulin-like growth factor (IGF) receptors [51] that promote hESC survival and pluripotency [52, 53]. Vitronectin-IGF-I function in hESC cultures is evident from a recent study showing that a synthetic vitronectin/IGF-I protein supports long-term hESC culture in the absence of feeder cells and serum [54].

Another receptor of interest, E-cadherin type I, was also down-regulated in hDF cells. E-cadherin-mediated cell-cell adhesion and signaling are essential for the colony formation and self-renewal of hESCs [55, 56] and a recombinant E-cadherin substratum has been used for feeder-independent culture of hESCs and hiPSCs [57]. Moreover, it has been postulated that E-cadherin -based cell contacts are stabilized by laminin-binding integrins possibly binding these two factors together in hESC signalling [58]. Surprisingly, KAL1 gene coding for Anosmin-1, was down-regulated 26-fold in hDF cells. Anosmin-1 enhances FGF signaling via regulation of bFGF/FGF receptor-1 (FGFR1)/heparin signaling -complex assembly and activity [59]. Anosmin-1 could thus play a role in maintaining a sufficient level of bFGF signaling in the hESCs.

Neither of the fibroblast feeder cell types secreted detectable amounts of bFGF in our ELISA analysis. In another study, hESC-supportive human fetal skin feeder cells have shown 3-fold up-regulation of bFGF compared to non-hESC supportive fetal feeder cells. However, only RNA levels

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were measured [40]. In a study comparing growth factor secretion of different fibroblast feeder cells by ELISA, the hFFs were found to secrete a few pg/ml bFGF, and also ActA with similar levels to our study [25]. The small discrepancies in the results are likely due to differing fibroblast culture and experimental conditions. The hESC-supportive hFF feeder cells were found to secrete more TGFß than the hDF feeder cells, but the addition of the same concentration of recombinant TGFß to hESC culture on hDFs did not rescue colony morphology or the level of TRA-1-81 expression. Partial rescue of hESC colony morphology and expression of TRA-1-81 near the level of hESC culture on hFFs, required a six times a week supplementation with tenfold concentration (6.0 ng/ml) of recombinant TGFß. It is thus unlikely that the difference between the feeder cells would derive from the difference in secretion of TGFß.

We conclude, that we have established xeno-free fibroblast feeder cell lines using HS-containing culture medium and, in accordance with published data agree, that HS is a feasible substitute for FBS [13, 17, 60-62]. We found several differences between xeno-free hESC-supportive and non-supportive feeder cells. We show that hESC-supportive feeder cells produce laminin-511, which may contribute to maintenance of hESC pluripotency. Our study also suggests that vitronectin and E-cadherin type I, both with known function in hESC biology, are likely to also have roles in feeder-dependent maintenance of pluripotency. Kallmann syndrome 1 sequence, coding for Anosmin-1, a known regulator of FGF signalling, is a novel candidate factor associated with hESC pluripotency through feeder cell culture. These findings provide clues for the development of defined culture conditions for human pluripotent stem cells.

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## 4. Materials and Methods

### 4.1 Cell lines and cell culture

#### 4.1.1. Derivation and culture of xeno-free fibroblasts

Five hDF cell lines: hDF 001/06, hDF 002/06, hDF 003/06, hDF 001/07, hDF 001/08 were derived from dermal tissues obtained from children under the age of 1 year in context of surgery. The tissues were donated with informed consent of voluntary parents, and the project had a supportive statement of the Ethics Committee of Pirkanmaa Hospital District (R05149). The fibroblast cell lines were derived as explant cultures similarly to previously published protocol [63]. Briefly, the tissue pieces were extensively washed with PBS (Lonza Group Ltd., Basel, Switzerland), subcutaneous tissue and epidermis were manually removed and dermis cut into 1 $mm^2$ pieces, placed on tissue culture dishes (Nalge Nunc International, Rochester, NY, USA) and covered with coverslips (Menzel-Gläser, Braunschweig, Germany). Culture medium (HS-medium) consisted of Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and 25 mM HEPES (Invitrogen Carlsbad, CA, USA), supplemented with 15% human serum (Type AB, PAA Laboratories GmbH Pasching Austria) and 0.5% Penicillin/streptomycin (Cambrex Bio Science, Walkersville, MD, USA). Human DF were cultured at 37 ºC, 5% $CO_2$, monitored daily for fibroblast outgrowth with Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V. Amstelveen, The Netherlands), and the culture medium was changed every 3-4 days. Upon confluency the fibroblasts were harvested and passaged with xeno-free, recombinant TrypLE Select (Invitrogen). Cells we cryopreserved at early passages in HS-medium supplemented with 5-10% Dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Human FF cells (CRL-2429) were purchased as a frozen stock (American Type Culture Collection, ATCC, Manassas, VA, USA) and thawn to HS-medium at passage level 5. The cell line was cultured up to passage level 13 and cryopreserved during the early passages in HS-medium supplemented with 5% DMSO. The hFFs were cultured in HS-medium and passaged with TrypLE Select similarly to hDFs.

Mouse EF cells were derived from pc ICR fetuses and cultured in 10% fetal bovine serum and 1X Glutamax in Dulbecco's Modified Eagle medium (all reagents from Invitrogen).

#### 4.1.2 Human embryonic stem cell lines

The two hESC lines used in this study, Regea 07/046 and Regea 08/017, were derived in Regea – Institute for Regenerative Medicine, University of Tampere, Finland. Both hESC lines were derived

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on hFF (CRL-2429) feeder cells and cultured and characterized as previously described [18]. Regea has approval of National Authority for Medicolegal Affairs Finland to study human embryos (Dnro1426/32/300/05) and a supportive statement of the Ethics Committee of Pirkanmaa Hospital District for the derivation, characterization, and differentiation of hESC lines (Skottman/R05116).

#### 4.1.3 Human embryonic stem cell culture on hFFs and hDFs

For the use as hESC feeder cells, the fibroblasts were inactivated by gamma irradiation (40 Gy), and 3.65 x $10^4$ cells/$cm^2$ were plated to culture dishes (Corning Incorporated, NY, USA). On day one after inactivation, adaptation to serum-free culture was performed by adding half HS-medium and half hESC medium. On day two, hESC medium was changed and hESCs were manually passaged on to the feeder cells. The hESC culture medium consisted of Knockout-Dulbecco's Modified Eagle‘s Medium (ko-DMEM) supplemented with 20% Knockout Serum Replacement (ko-SR), 2 mM GlutaMax (all from Invitrogen), 1 % MEM Eagle Non-Essential Amino acid solution (NEAA, Cambrex Bio Science), 50 U/ml penicillin/streptomycin (Cambrex Bio Science), 0.1mM ß-mercaptoethanol (Invitrogen) and 8 ng/ml bFGF (R&D Systems Minneapolis, MN, USA). For the testing of the influence of TGFß on hESC phenotype on hDF 001/08 feeder cells, recombinant human TGFß1 (PeproTech, London, UK) was added to parallel cultures of Regea 07/046 in hESC medium containing 8 ng/ml bFGF. TGFß concentrations of 0.6 ng/ml and 6.0 ng/ml were tested. Human ESCs were cultured and monitored as described above and culture medium was changed six times a week. Undifferentiated hESC colonies were manually passaged every 6 to 7 days onto new inactivated feeder cells.

### 4.2 Immunofluorescence

Regea 08/017 hESCs cultured on hFF CRL-2429 and hDF 001/08 were labelled with 1:200 dilution of anti-human Nanog antibody (R&D Systems) as described previously [7]. For laminin α5 chain immunofluorescence, hFF and hDF fibroblasts were cultured on 0.01% poly-L-lysine (Sigma-Aldrich) coated glass slides (BD Falcon) and fixed by 4% paraformaldehyde (PFA) (Sigma-Aldrich), for 15 min at room temperature (RT). The samples were washed by PBS, treated with 0.5% Triton X-100 in PBS for 5 minutes and blocked by Ultra V block solution (Thermofisher Scientific, Waltham, MA) for 10 minutes. All steps were performed at RT. The samples were incubated over night in the presence of the monoclonal antibody (clone 4C7) specific for human laminin α5 chain. After careful washing of the unbound antibody, the samples were incubated for 30 minutes with goat anti-mouse IgG AlexaFluor 488 that was diluted 1:500 in 0.1% Tween-PBS, at RT, in dark. Finally, the specimens were embedded in Vectashield Mounting Medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA). The images were acquired by

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Axioplan 2 fluorescence microscope, using Axiovision 4.8 software (Carl Zeiss Light Microscopy, Göttingen, Germany).

**4.3 Cell proliferation assay**

Cell proliferation of Regea 08/017 hESCs cultured on hFF CRL-2429 and hDF 001/08 feeder cells was determined using colorimetric immunoassay (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Mannheim, Germany) based on the measurement of BrdU incorporation during DNA synthesis. The hESC colonies were incubated overnight at 37ºC with 2x BrdU Labeling reagent, detached with TrypLE Select to single-cell suspension from the feeder cells and 1 x $10^4$ cells/well distributed to flat bottom 96-well plate (Nunc) with 8 replicates of each sample. The assay was performed according to manufacturer's instructions. The absorbance was measured at 450 nm with Viktor 1429 Multilabel Counter (PerkinElmer-Wallace, Norton, OH, USA). Cells without BrdU labelling were used as a background control. Inactivated fibroblasts alone were controlled for not incorporating BrdU. The analysis was repeated for a total of 4 times for hESCs on day 3, day 4 and day 5 after passaging at passage levels 1-3. The results were pooled and mean absorbance for background control was subtracted from the absorbance values. Pasw statistics 18 software and Mann–Whitney U-test were used to determine statistical difference between mean hESC proliferation rates on the two feeder types. P-values <0.05 and <0.01 were considered statistically significant and highly significant, respectively.

**4.4 Flow cytometry**

The hESC cultured on hFF CRL-2429 and hDF 001/08 were analyzed by flow cytometry for TRA-1-81 and OCT-3/4 expression at passages 1-6, 8 and 10. For hESCs cultured on hDF 001/08 flow cytometry analysis was conducted until the colonies had differentiated completely and no further passaging was feasible. Where TGFß growth factor was added to hDF 001/08 culture, the cells were analyzed for TRA-1-81 expression during passages 2-6. All hESC colonies without selection were collected for analysis and detached from the feeder cells to a single-cell suspension with TrypLE Select. For TRA-1-81 labelling, 0.1 x $10^6$ cells/sample were probed with 8 µl FITC Mouse anti-Human TRA-1-81 (Becton Dickinson, Franklin Lakes, NJ, USA) for 30 min at 4ºC in 0.5% BSA (Sigma-Aldrich) and 0.01% NaN3 (Sigma-Aldrich) in PBS. For OCT-3/4 labelling cells were fixed with 4% PFA for 10 min at RT and probed with 10µl Anti-human OCT-3/4-Fluorescein Monoclonal Antibody (R&D Systems) for 30 min at RT in 0.1% Saponin (Sigma-Aldrich) and 0.05% $NaN_3$ in PBS. The cells were analyzed using BD FACSAria (Becton Dickinson). The acquisition was set for 10.000 events per sample. PE-conjugated Goat F(ab´)2 Anti-Mouse IgG (Caltag Laboratories, Burlingame, CA, USA) and FITC Mouse IgM (Becton Dickinson) were used

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as isotype controls. The data were analyzed using FACSDiva Software version 4.1.2 (Becton Dickinson).

### 4.5 Q-PCR

#### 4.5.1 Analysis of pluripotency marker expression of hESCs

Regea 07/046 hESCs cultured on hFF CRL-2429 and hDF 001/08 were analyzed for the expression of pluripotency genes POU5F1 (OCT-3/4), NANOG, DNMTB3 and TDGF1 with q-PCR at passages 1-6 until complete hESC differentiation on hDF 001/08. All hESC colonies without any selection were manually cut off and collected for analysis.

The RNA was extracted with Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The RNA concentration and quality were assessed with NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 200 ng of RNA was translated to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The expression of the pluripotency genes were analyzed using Taqman gene expression assays (Applied Biosystems): Nanog (Hs02387400_g1), POU5F1 (Hs00999632_g1), TDGF1 (Hs02339496_g1) and DNMT3B (Hs01003405_m1). GAPDH (Hs99999905_m1) was used as a housekeeping control. The PCR reaction consisted of 3 µl of cDNA in 1:100 dilution, 7.5 µl of 2x TaqMan Universal PCR Master Mix (Applied Biosystems), 0.75 µl of assay and 3.75 µl of $H_2O$. All samples and no template controls were analyzed as three replicates. The q-PCR was carried out with Applied Biosystems 7300 Real-time PCR system: 2 min at 50ºC, 10 min at 95 ºC, and 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC.

The data was analyzed with 7300 System SDS Software (Applied Biosystems) and Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Relative quantification was calculated with the $-2^{\Delta\Delta Ct}$ method [64]. The data was normalized to the expression of GAPDH and the expression levels of Regea 07/046 cultured on hFF were used as reference level at each passage (fold change = 1). The data is presented as fold regulation values for better visualization of down-regulation: for fold change values greater than one, fold regulation is equal to fold change. For fold change values less than one, fold regulation is the negative inverse of the fold-change, calculated as -1/(fold change). Standard deviation of the 3 replicate fold change values is presented as error bars. For determining statistical significance, the mean fold regulation during the six culture passages on both feeder types was compared using the Mann–Whitney U-test.

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**4.5.2 ECM profiling of hFF and hDF fibroblasts**

For the comparison of the ECM profiles of the feeder cells, three biological replicates of both fibroblast types: hFF CRL-2429 at passages 9, 10 and 11 and hDF001/08 at passages 3, 4 and 5 were used for analysis. Feeder cells were prepared by γ-irradiation according to normal protocol. Cells were lysed for RNA extraction at day 3 post irradiation. The RNA was extracted with Qiagen RNeasy Plus Mini kit (Qiagen) according to manufacturer's instructions and concentrations assessed with NanoDrop 1000 spectrophotometer (NanoDrop Technologies). One µg of RNA from each sample was translated to cDNA with $RT^2$ First Strand Kit (SABiosciences, MD, USA) according to manufacturer's instructions. The cDNA was loaded to $RT^2$ Profiler™ PCR Array Human Extracellular Matrix and Adhesion Molecules –array (PAHS-013A) according to manufacturer's instructions. All reagents were bought from SABiosciences. The q-PCR was carried out with Applied Biosystems 7300 Real-time PCR system: 10 min at 95ºC, 40 x 15 sec at 95ºC and 1 min at 60ºC, followed by the melting curve program: 15 sec at 95ºC; 1 min at 60ºC; 15 sec at 95ºC; 15 sec at 60ºC.

The data was analyzed with $RT^2$ Profiler™ PCR Array Data Analysis software (http://www.sabiosciences.com/pcr/arrayanalysis.php) using the $-2^{\Delta\Delta Ct}$ -based fold-change calculations and normalizing the data with the expression of the housekeeping genes Beta-2-microglobulin (B2M), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), Ribosomal protein L13a (RPL13A), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Actin, beta (ACTB). The expression levels of hFF CRL-2429 were used as reference level for each gene. Fold regulation was calculated as described above. Statistical analysis (Student's t-test) was conducted by the software.

**4.5.3 Analysis of laminin subunit α4, α5, γ2 and B-CAM expression of hFF and hDF fibroblasts**

Human FF CRL-2429 (passages 9, 10 and 11) and hDF001/08 (passages 3, 4 and 5) feeder cells were additionally analyzed for laminin chain α4, α5, γ2 and B-CAM expression with Taqman gene expression assays: LAMA4, Hs00158588_m1; LAMA5, Hs00245699_m1; LAMC2, Hs01043711_m1; BCAM, Hs00170663_m1. GAPDH (Hs99999905_m1) was used as a housekeeping control. RNA extraction, cDNA synthesis and q-PCR reactions were conducted as described above in 4.5.1., except 1:5 dilution of cDNA was used for the q-PCR reactions. Expression levels of hFF CRL-2429 were used as reference level.

**4.6 Radioactive, metabolic labelling and immunoprecipitation of the culture media**

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The metabolic labelling, sample preparation and detection were performed as described in detail in [45]. Briefly, 100 μCi of $^{35}$S-labelled methionine was added to the methionine-deprived culture medium and incubated overnight. The labelled culture media were collected, preabsorbed with GammaBindSepharoseBeads and then absorbed over night with GammaBindSepharoseBeads pre-coupled with antibodies directed to laminin α5 chains. In case of human fibroblasts, we used mAB, clone 4C7, directed against human laminin α5 chain and in case of mEFs, we used pc antibody, clone 1121, that recognizes mouse laminin-511 (the latter was a kind gift from Dr. Takako Sasaki). After an overnight immunoprecipitation, the bound proteins were washed, eluted to Laemmli's buffer and analyzed by SDS-PAGE. JAR choriocarcinoma cells (HTB-144, ATCC) synthesizing laminin-511 were used as positive control cell line. For the negative control precipitations, the antibody was omitted.

**4.7 Growth factor secretion analysis**

Human FF CRL-2429 and hDF 001/08 feeder cell CM were analyzed for bFGF, ActA and TGFß growth factor secretion with enzyme-linked immunosorbent assay (ELISA). The feeder cells were prepared by irradiation as described above. On days 4-7 post inactivation, 0.2 ml/cm$^2$ of hESC medium without bFGF was collected daily after overnight incubation on the feeder cells and stored at -70ºC prior the ELISA analysis. Following ELISA kits were used: Quantikine Human FGF basic Immunoassay (DFB50), Quantikine Human TGF-ß1 Immunoassay (DB100B) and Quantikine Human/Mouse/Rat Activin A Immunoassay (DAC00B), all from R&D Systems. CM were analyzed according to manufacturer's instructions. All media samples were analyzed as duplicates and measurements repeated twice with similar results. Mann–Whitney U-test was used for statistical analysis.

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**5. Acknowledgements**

We thank Outi Melin, Hanna Koskenaho, Elina Konsén, Niina Ikonen, and Pipsa Kaipainen for technical assistance. The work was supported by: TEKES, the Finnish Funding Agency for Technology and Innovation (Dnro 1128/31/05), the Academy of Finland (218050), The Competitive Research Funding of the Tampere University Hospital (Grants 9G107 and 9H114), The Finnish Cultural Foundation, Tampere Graduate Program in Biomedicine and Biotechnology and Helsinki Biomedical Graduate School.

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## 6. Figure legends

**Figure 1** Overview of the workflow.

**Figure 2** Characterization of hESCs cultured on hFF CRL-2429 and hDF 001/08 feeder cells. (A) Typical colony morphology of Regea 08/017 and immunostainings for Nanog in the beginning of culture and after serial passaging. Colonies on hFFs showed undifferentiated morphology and stable Nanog expression, whereas on hDFs hESCs differentiated with corresponding changes in morphology and weaker expression of Nanog. Scale bars 500 µm for phase contrast images and 200 µm for fluorescence images. (B) Cell proliferation analysis showed higher proliferation rate for Regea 08/017 cultured on hFF feeder cells compared to those cultured on hDFs (*p= 0.003, Mann–Whitney U-test). (C) Relative q-PCR analysis of Regea 07/046 cultured on the two feeder cell types showed progressive down-regulation of all four pluripotency markers analyzed for hESCs cultured on hDFs compared to hESCs cultured on hFFs, set as reference =1. The down-regulation of all genes was statistically highly significant (p-values = 0.004, Mann–Whitney U-test). (D) Flow cytometry analysis for Regea 07/046 cultured on hFFs and hDFs over 10 passages. hESCs showed constant expression of both TRA-1-81 and OCT-3/4 on hFFs, but progressive differentiation with decreasing positivity for both markers on hDFs. End point analysis shown in (E) and (F), black = negative control, blue = isotype control.

**Figure 3 (**A) Volcano plot for ≥ 2-fold down-regulated (green) and ≥ 2-fold up-regulated (red) ECM and related genes for hDF 001/08 compared to hFF CRL-2429 feeder cells analyzed with RT² Profiler™ PCR Array. Genes above the blue line have a p-value < 0.05 (student's t-test, RT² Profiler™ PCR Array Data Analysis software, SABiosciences). (B) Integrin and laminin subunit fold regulations for hDFs compared to hFFs analyzed by q-PCR.

**Figure 4** Laminin α5 chain production by the hFF, mEF and hDF feeder cell lines. A) The cells were radioactively labelled followed by immunoprecipitation and SDS-PAGE analysis. hESC-supportive hFF and mEF feeder cells synthesize and secrete laminin α5 chain, whereas none of the hDF cell lines do. Positive laminin α5 bands shown by arrows. JAR = positive control cells. -Ab: no primary antibody, negative control precipitation. B) Indirect immunofluorescence staining with monoclonal antibody specific for human laminin α5 chain confirms that hFF cells synthesize laminin α5 (white arrow). The hDF lines show no laminin α5 expression. Lower panel shows the negative control stainings carried out without primary antibody (No MAb). 40X magnification.

**Figure 5** Growth factor secretion analyses of hFF CRL-2429 and hDF 001/08 feeder cells on day 4 to 7 after irradiation. (A) No statistical difference in average secretion of ActA between the two feeder cells. (B) hFFs secreted in average 0.6 ng/ml more TGFß compared to hDFs. The difference was statistically highly significant (p<0.01, Mann–Whitney U-test). The Knockout Serum Replacement (ko-SR) was measured to contain 315 pg/ml TGFß and hESC medium (20% ko-SR) thus calculated to contain approximately 63 pg/ml TGFß, marked in the picture with a black line. (C) Regea 07/046 colony morphology after 5 passages of TGFß supplementation to culture on hDFs. Addition of 0.6 ng/ml TGFß did not rescue hESC colony morphology, whereas 6.0 ng/ml TGFß resulted in more compact colonies with improved morphology. Scale bars 500 µm. (D) Flow cytometry analyses during TGFß supplementation at passages 2-6. Average percent of TRA-1-81 positive hESCs cultured on hFFs in hESC medium and on hDFs in hESC medium as well as in hESC medium supplemented with TGFß are shown.

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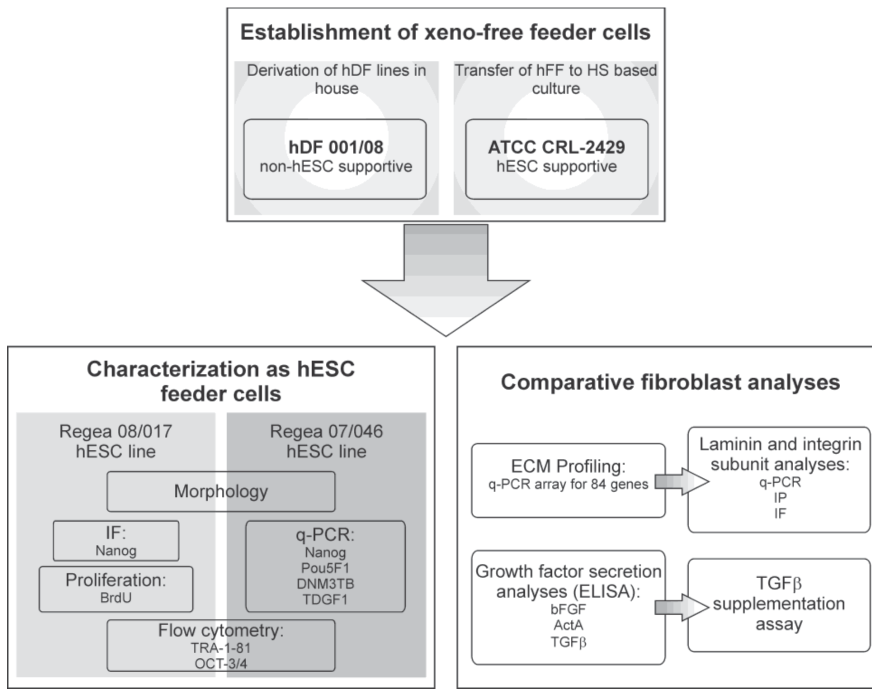


Fig. 1

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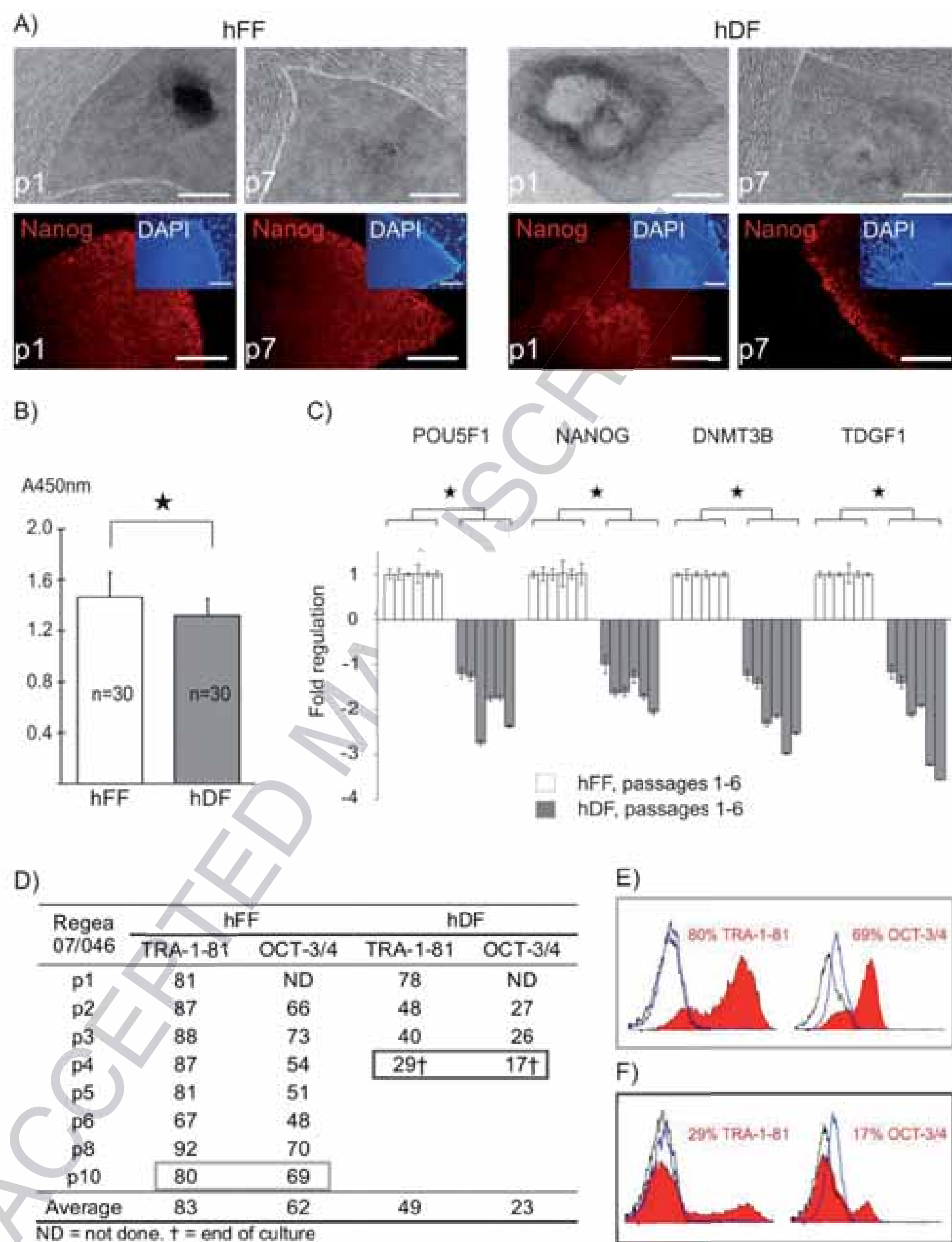


| Regea 07/046 | hFF | | hDF | |
|---|---|---|---|---|
| | TRA-1-81 | OCT-3/4 | TRA-1-81 | OCT-3/4 |
| p1 | 81 | ND | 78 | ND |
| p2 | 87 | 66 | 48 | 27 |
| p3 | 88 | 73 | 40 | 26 |
| p4 | 87 | 54 | 29† | 17† |
| p5 | 81 | 51 | | |
| p6 | 67 | 48 | | |
| p8 | 92 | 70 | | |
| p10 | 80 | 69 | | |
| Average | 83 | 62 | 49 | 23 |

ND = not done. † = end of culture

**Fig. 2**

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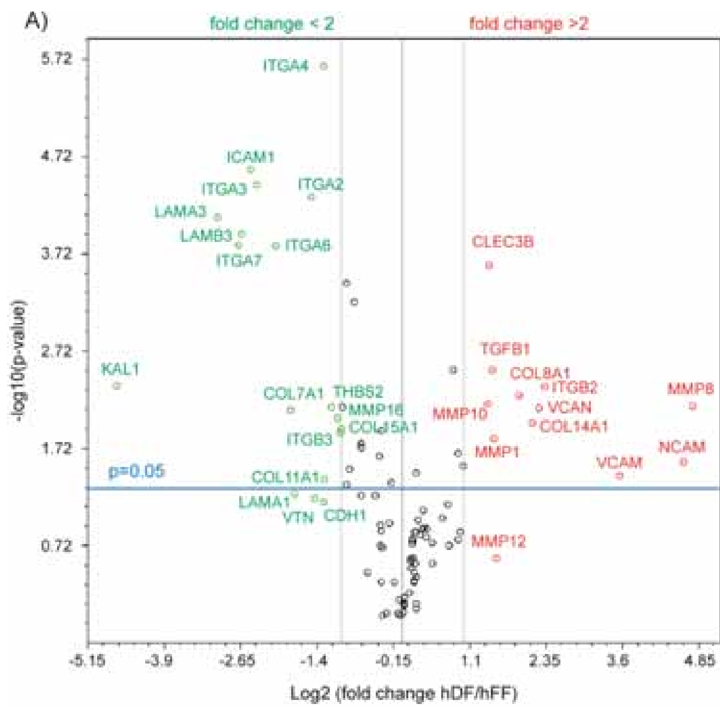


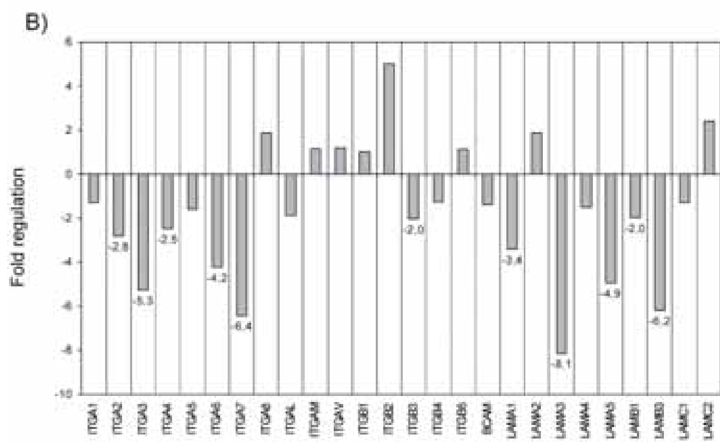


**Fig. 3**

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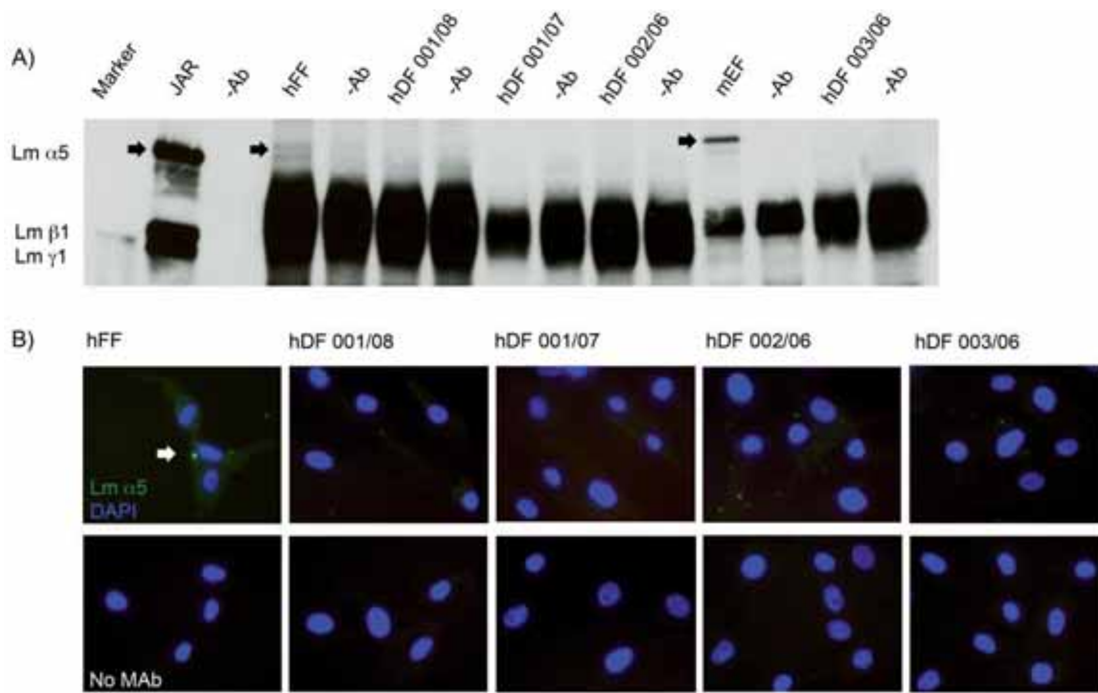


Fig. 4

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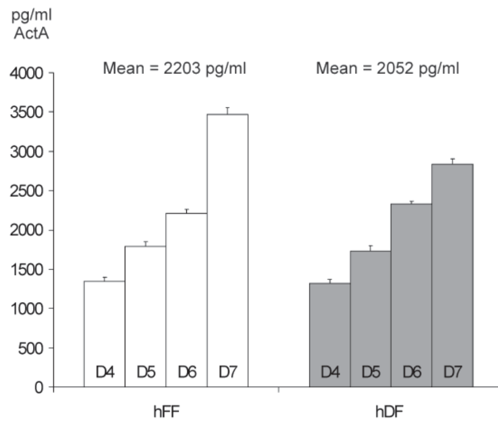
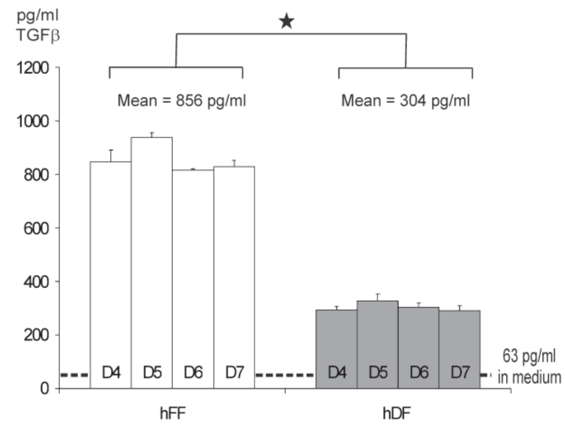
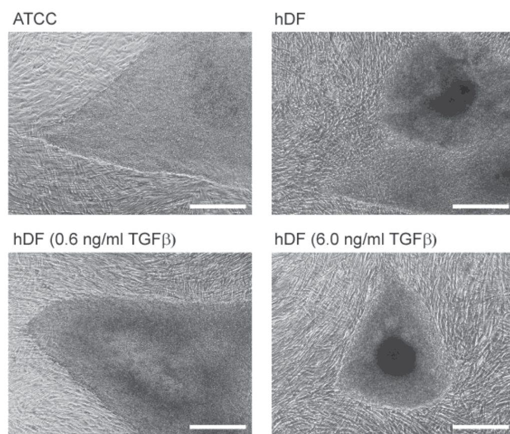
A)




B)




C)




D)

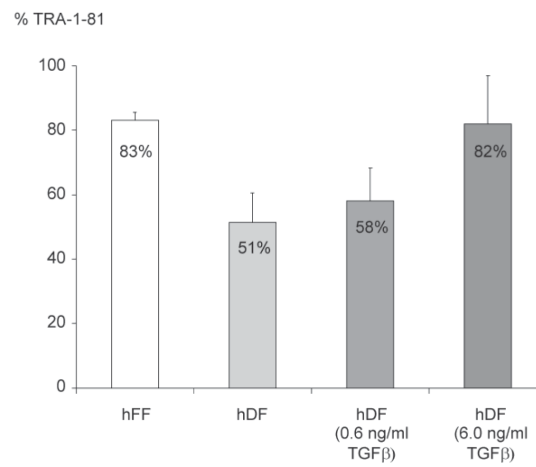



**Fig. 5**

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Vol.2, No.4, 176-186 (2012)
doi:10.4236/scd.2012.24022

Stem Cell Discovery

# Low level of activin A secreted by fibroblast feeder cells accelerates early stage differentiation of retinal pigment epithelial cells from human pluripotent stem cells

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Received 6 July 2012; revised 3 August 2012; accepted 3 September 2012

## ABSTRACT

**Human pluripotent stem cells (hPSC) differentiated to retinal pigment epithelial cells (RPE) provide a promising tool for cell replacement therapies of retinal degenerative diseases. The *in vitro* differentiation of hPSC-RPE is still poorly understood and current differentiation protocols rely on spontaneous differentiation on fibroblast feeder cells or as floating cell aggregates in suspension. The fibroblast feeder cells may have an inductive effect on the hPSC-RPE differentiation, providing variable signals mimicking the extraocular mesenchyme that directs the differentiation *in vivo*. The effect of the commonly used fibroblast feeder cells on the hPSC-RPE differentiation was studied by comparing suspension differentiation in standard RPEbasic (no bFGF) medium to RPEbasic medium conditioned with mouse embryonic (mEF-CM) and human foreskin (hFF-CM) fibroblast feeder cells. The fibroblast secreted factors were found to enhance early hPSC-RPE differentiation. The onset of pigmentation was faster in the conditioned media (CM) compared to RPEbasic for both human embryonic (hESC) and induced pluripotent (iPSC) stem cells, with the first pigments appearing around two weeks of differentiation. After four weeks of differentiation, CM conditions consistently contained higher number of pigmented cell aggregates. The ratio of PAX6 and MITF positive cells was quantified to be clearly higher in the CM conditions, with mEF-CM containing most positive cells. The mEF cells were found to secrete low levels of activin A growth factor that is known to regulate eye field differentiation. As RPEbasic was supplemented with corresponding, low level (10 ng/ml) of recombinant human activin A, a clear increase in the hPSC-RPE differentiation was achieved. Thus, inductive effect provided by feeder cells was at least partially driven by activin A and could be substituted with a low level of recombinant growth factor in contrasts to previously reported much higher concentrations.**

**Keywords:** Retinal Pigment Epithelial Cell; Human Pluripotent Stem Cell; Conditioned Medium; Human Foreskin Fibroblast; Mouse Embryonic Fibroblast; Activin A; Cell Differentiation

## 1. INTRODUCTION

Retinal pigment epithelium (RPE) is a highly polarized and specialized monolayer of cells located between the neural retina and choroid at the back of the eye. RPE has several vitally important functions as a part of the blood-retina-barrier and in supporting photoreceptor function and survival [1,2]. RPE degeneration has a major role in pathogenesis of retinal diseases including age-related macular degeneration (AMD) and retinitis pigmentosa. The degeneration of RPE cells leads to the degradation of photoreceptors and as a consequence to either partial or total loss of vision. Currently, functionality of destroyed RPE cells can be restored only with cell transplantation, setting high demands to develop novel cell sources for replacement therapy. Transplantation of RPE cells has been studied extensively in animal models and also in humans [2-5]. Several cell sources have been

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studied for cell therapy but currently human pluripotent stem cells (hPSCs) are considered to be the most promising cell source of differentiating cells for tissue engineering applications due to their differentiation potential and high replicative capacity. Several research groups have reported successful differentiation of RPE cells from hPSCs [6-10] and first clinical studies using human embryonic stem cell (hESC) derived RPE cells are ongoing [11].

During mammalian development, RPE and neural retina are both derived from optic neuroepithelium and share the same progenitor [12]. The neuroepithelium near the anterior part of the neural tube evaginates laterally to form the optic vesicles. Invagination of the distal part of the optic vesicle leads to the formation of the optic cup in a complex environment affected by many external signals [13]. By the sixth or seventh week of development, the optic cup has differentiated into two epithelial sheets. Of these, the distal layer differentiates into the neural retina and the proximal layer develops into the RPE in interactions with the surrounding extraocular tissue, including the extraocular mesenchyme [12,14,15].

In the absence of external signal molecules, the hPSCs choose the neural differentiation pathway as a default [16]. Most of the published hPSC-RPE differentiation protocols rely on spontaneous differentiation in absence of basic fibroblast growth factor (bFGF). The induction of differentiation is based on confluent overgrowth on feeder cells especially mouse embryonic fibroblasts (mEF) or through embryoid body/neurospehere formation [7,9,10]. Recently, RPE differentiation efficiency has been enhanced with prolonged culture and growth factor/inhibitor based differentiation strategies. Factors, such as activin A, transforming growth factor $\beta$1 (TGF$\beta$1), and nodal antagonist SB431542 [17] as well as Wnt signaling inhibitor CKI-7 together with Dkk-1, Lefty-A, FGF antagonist Y-27632 and SB431542 [18,19] have been used. Regardless of these, many groups are using feeder cell (mEF, foreskin fibroblasts, PA6 cells) containing methods with spontaneous differentiation method [20,21] and first clinical studies are conducted with mEF supported and spontaneously differentiated hESC-RPE cells [11]. It is not clear why the removal of FGFs from feeder cell based hPSC cultures [20] or the use of PA6 stromal feeder cell to promote neural differentiation [22-24] is sufficient to produce RPE cells but both of the differentiation strategies suggest important function of external signals provided by mesenchymal fibroblasts/stromal cells.

We hypothesized that fibroblast feeder cells used for the culture of undifferentiated hPSC may have an inductive effect on RPE cell differentiation providing mesenchymal signals necessary for the key cellular decision guiding optic cup differentiation and further cell commitment towards RPE cell fate [25,26]. Moreover we hypothesized that different feeder cells types (mEF and human foreskin fibroblast, hFF) may provide variable mesenchymal signals guiding RPE differentiation. In this study, we studied the inductive effects of feeder cells routinely used for hPSC differentiation towards RPE cells. Human PSCs were differentiated using media conditioned by two types of fibroblasts feeder cells (hFF-CM and mEF-CM) and non-conditioned differentiation medium (RPEbasic).

## 2. MATERIALS AND METHODS

All cells were cultured in 37˚C, 5% $CO_2$ incubator (Thermo Electron Corp., Waltham, MA, USA) and monitored regularly with Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands).

### 2.1. Fibroblast Feeder Cell Culture

Human FF (CRL-2429™, American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria) and 0.5% Penicillin/Streptomycin (Lonza Group Ltd, Basel, Switzerland). P-MEF (EmbryoMax®, Millipore, Billerica, MA, USA) were cultured in Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 10% FBS and 1% GlutaMax-I, sterile-filtered prior use. Cell culture flasks for mEF were precoated with 0.1% porcine gelatin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature (RT). Both fibroblast cell lines were purchased as frozen stocks and cryopreserved at early passages with 5% - 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) supplementation.

### 2.2. Human Pluripotent Stem Cell Culture

The human embryonic stem cell (hESC) line Regea 06/040 was derived at IBT—The Institute of Biomedical Technology (former Regea—Institute for Regenerative Medicine), University of Tampere, Finland. The hESC line was derived on hFF feeder cells and cultured and characterized as described previously [27]. Human induced pluripotent stem cell (iPSC) line FiPS5-7 was established by Professor Otonkoski's research group at University of Helsinki, Finland. It was generated from human fibroblasts using four transcription factors—*OCT*3/4 (*POU*5*F*1), *SOX*2, *nanog*, and *LIN*28 [28], and transgene silencing was confirmed with qPCR [29]. Prior to the experiments, both pluripotent cell lines were cultured on hFF feeder cells in standard hPSC culture medium consisting of KO-DMEM supplemented with 20%

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Knock-Out Serum Replacement (KO-SR), 2 mM GlutaMax-I, 0.1 mM 2-mercaptoethanol (all from Life Technologies), 1% Non-Essential Amino Acids (NEAA), 50 U/ml Penicillin/Streptomycin (both from Lonza Group Ltd.) and 8 ng/ml human bFGF (R&D Systems Inc., Minneapolis, MN, USA). The culture medium was changed five times a week and undifferentiated colonies were manually passaged onto new, $\gamma$-irradiated (40 Gy) feeder cell layers once a week.

## 2.3. Collection of Conditioned Media

Both hFF (passage 6-11) and mEF (passage 4-5) were harvested at confluency with TrypLE™ Select (Life Technologies) at 37˚C, 15 min, and mitotically inactivated with $\gamma$-radiation (40 Gy). Irradiated fibroblasts were seeded onto 0.1% gelatin-coated culture dishes (cell density $3.6 \times 10^4/cm^2$) and left to adhere overnight. The cells were adapted to serum-free culture conditions by sequential addition of RPE differentiation medium (RPEbasic) the day after irradiation. RPEbasic included the same reagents as described above for hPSC culture medium, but supplemented with 15% KO-SR and lacking bFGF. For a period of 10 days, 2 ml/cm$^2$ of RPEbasic was collected daily from the culture dishes and replaced with equal amount of fresh medium. Collected media were centrifuged at 1000 rpm, 4 min, transferred to new tubes and stored at −70˚C. After collection, CM for each fibroblast type was thawed, pooled, and stored at −70˚C in aliquots until used for differentiation experiments. Four different batches of CM were similarly prepared for both fibroblast types.

## 2.4. Differentiation Culture

Undifferentiated hPSC colonies (Regea06/040 and FiPS5-7) were manually dissected, and the pieces transferred to low cell-bind cell culture plates (Corning Inc., Corning, NY, USA) in RPEbasic, mEF-CM or hFF-CM. The media were changed five times a week. Human ESC line (Regea06/040) was used for differentiation experiments at passage levels 31 - 91 and hiPSC line (FiPS5-7) at passage levels 48 - 117. The differentiation experiments were repeated six times in total. Influence of activin A on RPE differentiation was tested with hESC line (Regea06/040) (passages 37 - 42) using RPEbasic supplemented with 10 ng/ml activin A (Peprotech, London, England). The activin A supplementation test was repeated three times. The workflow of the study and analyses performed are summarized in **Figure 1**.

After six to seven weeks in suspension culture, pigmented areas of cell aggregates were selectively replated to adherent cultures, in order to create purified populations of hPSC-RPE. Pigmented areas were selected, washed with Dulbecco's Phosphate Buffered Saline (DPBS, Lonza Group Ltd.) and dissociated with 1× Trypsin-EDTA (Lonza Group Ltd.) for 20 - 35 min at 37˚C with repeated trituration. Trypsin was inactivated with 10% human serum (PAA Laboratories), and cells collected to appropriate culture medium through 40 µm cell strainers. Dissociated cells were plated either on 24-well plate wells (Corning Cellbind, Corning Inc.) coated with 5 µg/cm$^2$ human placental collagen IV (Sigma-Aldrich) for 3 h at 37˚C, or to permeable 0.3 cm$^2$ BD BioCoat™ mouse collagen IV cell culture inserts (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Adherent cultures were maintained using appropriate media that were changed three times a week.

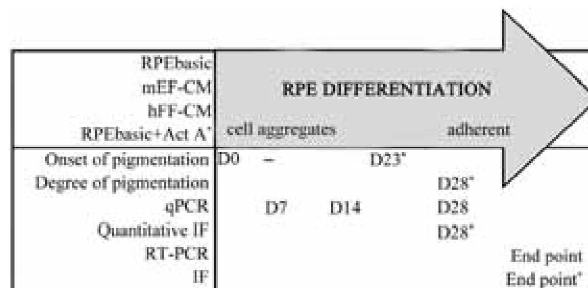


**Figure 1.** Workflow of the study. RPE differentiation was performed using four test media: RPEbasic, hFF and mEF conditioned media and RPEbasic supplemented with activin A. Analyses performed in different time points are presented.

## 2.5. Analysis of Pigmentation

The onset of pigmentation was followed daily and the day of appearance of the first pigmented cells in each medium was recorded. The appearance of first pigmentation was recorded from five individual differentiation experiments for hESCs, four experiments for hiPSCs, and three activin A supplementation experiments. To assess the amount of pigmentation after four weeks of differentiation, the ratio of cell aggregates containing pigment in relation to total number of aggregates was counted after 28 days of differentiation. This was done in three individual differentiation experiments for both studied cell lines and for all three activin A supplementation experiments. Results were plotted using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4.

## 2.6. Quantitative Real-Time Polymerase Chain Reaction

Differences in expression levels of genes related to RPE differentiation: retina and anterior neural fold homeobox (*RAX*), paired box gene 6 (*PAX*6) and microphthal-mia-associated transcription factor (*MITF*), were studied with qPCR. Gene expression was evaluated for hPSCs differentiated in the three test media: RPEbasic, mEF-CM and hFF-CM, after 7, 14, and 28 days in

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differentiation culture. Additionally the expression of neural retina markers ceh-10 homeodomain containing homolog (*CHX*10) and cone-rod homeobox protein (*CRX*) was studied after 28 days of differentiation.

Ten to fifteen differentiated cell aggregates were collected from each test medium. In addition, pieces of undifferentiated colonies of both hPSC lines were collected for control material prior to the beginning of the experiment. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, GmbH & Co., Düren, Germany), according to the manufacturer's protocol. The RNA quality and concentration were determined using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 200 ng of each RNA sample, using MultiScribe Reverse Transcriptase in the presence of RNase inhibitor (High-capacity cDNA RT kit, Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. The synthesis of cDNA was carried out in PCR MasterCycler (Eppendorf AG, Hamburg, Germany): 10 min at 25˚C, 120 min at 37˚C, 5 min at 85˚C, and finally cooled down to 4˚C.

FAM-labeled TaqMan® Gene Expression Assays (Applied Biosystems Inc.) were used for qPCR reactions: *RAX* (Hs00429459_m1), *PAX6* (Hs00240871_m1), *MITF* (Hs01115553_m1*),* *CRX* (Hs01549131_m1) and *CHX*10 (Hs01584048_m1). Glyceraldehyde 3-phosphate dehydrogenase, *GAPDH* (Hs99999905_m1) was used as endogenous control. Each reaction mixture consisted of 7.5 µl TaqMan® Universal PCR Master Mix (2x), 0.75 µl Gene Expression Assay (20x), 3 µl of cDNA (diluted 1:5 with sterile water) and sterile water to the total volume of 15 µl. All samples and controls were run as triplicate reactions using the 7300 Real-time PCR system (Applied Biosystems Inc.) as follows: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 s at 95˚C, and 1 min at 60˚C. Results were analyzed using 7300 System SDS Software (Applied Biosystems Inc.). Based on the $C_T$-values given by the software, the relative quantification of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [30] and Microsoft Excel 2003.

The values for each sample were normalized to expression levels of *GAPDH*. The expression level of undifferentiated hPSC sample was set as the calibrator (fold change equals 1). Results were plotted using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4. For visualization of down-regulation, the fold change values < 1 are presented as the negative inverse of the value, calculated as –1/(fold change). Standard deviations were calculated for each set of technical replicates, and presented as error bars.

## 2.7. Immunofluorescence

Differences in protein expression of PAX6 and MITF after 28 days of differentiation were studied using immunofluorescence. The cell aggregates were dissociated to single cells as described above. Single-cell suspensions containing 1.6 - 3.5 × $10^5$ cells/ml were prepared in DPBS, and 150 µl samples were centrifuged onto 15-mm glass cover slips at 600 rpm, 5 min, using Shandon Cytospin 2 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Cells were fixed immediately with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min at RT. Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in DPBS at RT for 10 min, and unspecific binding blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) at RT for 1 h. Incubation with primary antibodies was carried out either overnight at 4˚C or for 1 h at RT with the appropriate antibody: 1:200 dilution of mouse anti-PAX6 (Developmental Studies Hybridoma Bank, University of IOWA, Department of Biology, Iowa City, IA, USA) or 1:350 dilution of rabbit anti-MITF (Abcam, Cambridge, UK). Secondary antibodies were diluted 1:1500 in 0.5% BSA-DPBS and cells incubated 1 h at RT in either Alexa Fluor 568-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (both from Molecular probes, Life Technologies). Cell nuclei were stained with VectaShield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) containing 4', 6'-diamidino-2-phenylidole (DAPI). Cells were imaged with Olympus BX60 microscope (Olympus, Tokyo, Japan) using a 40× objective. The images were captured using same exposure time within each experiment and imaged areas were selected randomly. Minimum 700 cells were counted from each condition. PAX6 and MITF expression was quantified using Image J Image Processing and Analysis Software [31]. For each experiment, the threshold for positive expression was set by analysing several randomly selected images. Intensity threshold was adjusted for each image within an experiment and label to normalize the levels of background intensity. Cells below the set threshold level were considered negative. The total number of cells in each image was determined by counting nuclei counterstained with DAPI. The numbers of cells expressing PAX6 or MITF in relation to the total amount of cells were counted for hESC-RPE from two individual experiments and two activin A supplementation experiments. Results were plotted as bar charts using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4.

Monolayers of hESC-RPE maturated on mouse collagen IV cell culture inserts were analyzed with immunofluorescence for the expression and correct localization of RPE-related proteins: MITF, cellular retinaldehyde-binding protein (CRALBP), Bestrophin, and tight junction protein zona occludens-1 (ZO-1). Detailed protocol has been published previously [21]. Images were taken either with Olympus BX60 microscope or LSM 700 confocal microscope (Carl Zeiss, Jena, Ger-

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many) using a 63× oil immersion objective. All images were edited using ZEN 2009 Light Edition (Zeiss) and Adobe Photoshop CS4.

## 2.8. Reverse Transcriptase PCR

Monolayers of hPSC-RPE maturated on human collagen IV were analyzed for expression of RPE specific genes by reverse transcription polymerase chain reaction (RT-PCR). Expression of the following genes was assessed: RPE precursor markers *MITF* and orthodenticle homeobox 2 (*OTX*2), and mature RPE-specific markers retinal pigment epihelium-specific protein 65 kDA (*RPE*65), bestrophin (*BEST*1), pre-melanosomal protein 17 (*PMEL*17), pigment epithelium-derived factor (*PEDF*) and tyrosinase (*TYR*). *GAPDH* was used as endogenous control. Total RNA was extracted and 40 ng was reverse-transcribed to cDNA as described above. Genomic control reactions excluding the reverse transcriptase enzyme (-RT) for each RNA sample were performed. RT-PCR was carried out using 1 μl of cDNA as template. Detailed protocol and primer sequences used have been previously published [21].

## 2.9. Growth Factor Secretion Analysis

The three differentiation media: RPEbasic, mEF-CM and hFF-CM were analyzed for concentrations of TGF-$\beta$1, activin A and bFGF growth factors with enzyme-linked immunosorbent assay (ELISA). The following commercial ELISA kits were used: Human TGF-$\beta$1 Immunoassay, Human/Mouse/Rat Activin A Immunoassay, human FGF basic Immunoassay (all from Quantikine®, R&D Systems, Minneapolis, MN, USA). The Human TGF-$\beta$1 Immunoassay and human FGF basic Immunoassays have been previously shown to detect the growth factor concentrations also from mEF-CM [32]. All assays were performed according to manufacturer's instructions. All standards and samples were tested in duplicates. For the activin A immunoassay, each sample was diluted 1:5 and 1:25, with the diluent supplied in the kit. Optical densities were measured using Wallac Victor2™ 1420 Multilabel counter (Perkin Elmer-Wallace, Norton, OH, USA). Using optical densities of the standard series, standard curves were created using Microsoft Excel 2003 and concentrations of the samples calculated accordingly. Standard deviations were calculated from the concentrations of duplicates of each tested sample, and were presented as error bars. The measurements were repeated twice from two different batches of CM.

## 2.10. Ethical Considerations

The study of human embryos at University of Tampere has been approved by National Authority for Medicolegal Affairs Finland (TEO) (Dnro 1426/32/300/05). We have a supportive statement of Ethical Committee of Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines from surplus human embryos (Skottman/R05116). No new cell lines were derived for this study.

# 3. RESULTS

## 3.1. Appearance of Pigmentation Was Accelerated in the Conditioned Media

Human ESCs and iPSCs were differentiated in suspension as floating cell aggregates in three different media: standard RPEbasic, hFF-CM and mEF-CM. Differentiation rate of hPSC-RPE was monitored by recording the appearance of first pigmented cells in each medium. The appearance of pigmented cells was faster in the CM compared to the RPEbasic for both hESCs and iPSCs. On average, the hESCs pigmented fastest in mEF-CM (day 13), next in hFF-CM (day 15) and slowest in RPEbasic (day 16) (**Figure 2A**). Human iPSCs generated pigmented cells on average at day 16 in both CM and at day 18 in RPEbasic (data not shown).

## 3.2. hPSCs Expressed Marker Genes for Eye Field and RPE Precursor Cells during Differentiation

Gene expression of early eye field markers *PAX6* and *RAX*, and RPE precursor marker *MITF* was analyzed with relative qPCR after 7, 14 and 28 days of differentiation. The gene expression levels were compared to undifferentiated hPSCs. For hESCs, expression of *PAX6* increased substantially during differentiation, suggesting that differentiation progressed to eye field direction in all studied media (**Figure 2(B)**). Expression levels of *RAX* increased during the first two weeks of differentiation, and decreased by day 28 in cells differentiated in both CM (**Figure 2(C)**). This decrease in *RAX* expression was accompanied by a 10-fold increase in the expression of RPE-specific *MITF* (**Figure 2(D)**) which indicates progress toward RPE fate. In RPEbasic, expression of *RAX* further increased by day 28, but the pattern of *MITF* expression was similar to that of CM. In addition, the expression of neural retina markers *CHX10* and *CRX* were analyzed at day 28 and found to be substantially decreased for both CM conditions compared to RPEbasic. Especially the early neural retina marker CHX10 expression was 15 times higher in the RPEbasic condition compared to mEF-CM and 9 times higher compared to hFF-CM (data not shown). This expression pattern indicated increased differentiation toward neural retina direction in RPEbasic and toward RPE fate in CM. The studied genes showed a similar expression pattern also in

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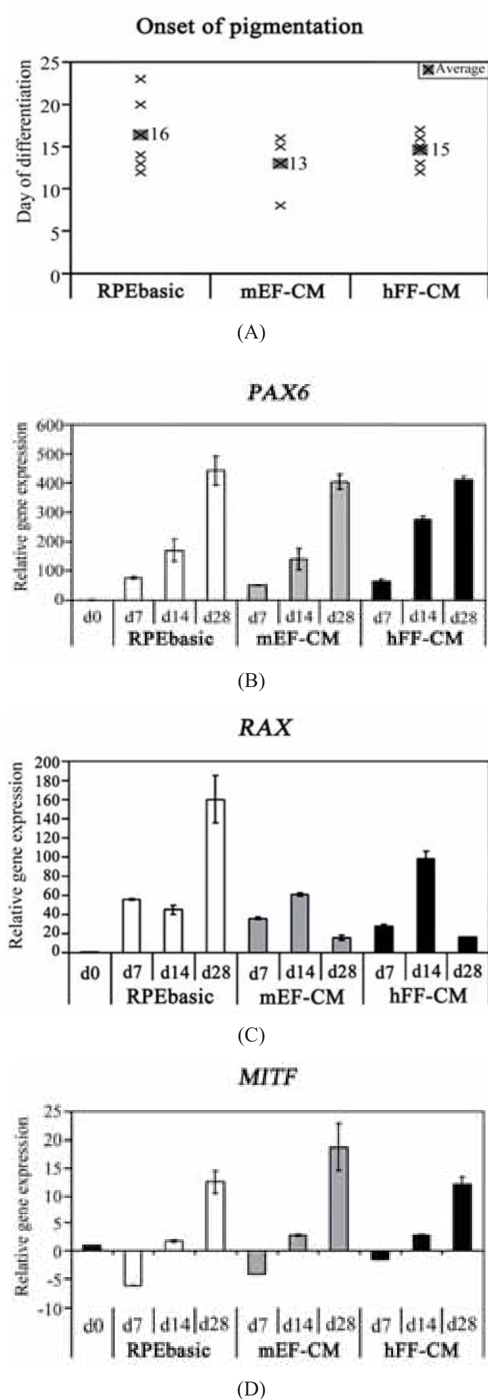


**Figure 2.** Analysis of early-stage hESC-RPE differentiation. The day of first pigmentation observed in replicate experiments, as well as the average, shown for each medium (A). Gene expression of the early eye-field markers *PAX*6 (B) and *RAX* (C) as well as early RPE marker *MITF* (D), relative to undifferentiated stem cells (d0) was analysed with qPCR.

hiPSC differentiation but with lower relative expression levels (data not shown).

## 3.3. Conditioned Media Contained More RPE Cells after Four Weeks of Differentiation

After four weeks of differentiation, the number of pigmented cell aggregates to total number of aggregates was calculated for each medium. CM consistently contained higher percentage of pigmented cell aggregates compared to RPEbasic for both hESCs (**Figure 3(A)**) and hiPSCs (data not shown) in each of the three replicate experiments. Typically, the pigmented areas were also larger in CM compared to RPEbasic (**Figure 3(B)**), indicating higher number of pigmented cells within the areas. In addition, the number of PAX6 and MITF expressing cells in the differentiated cell aggregates in each medium were quantified at 28 day time point. After dissociation to single cells and immunostaining, the number of positive cells was calculated. In two replicate experiments, the ratios of PAX6 and MITF positive cells were clearly higher in CM compared to RPEbasic (**Figure 3(C)**) with mEF-CM containing highest percentage of positive cells. On average, over 90% of cells expressed PAX6 and MITF in both CM, whereas in RPEbasic only 61% (±8%) of cells were positive to PAX6 and 74% (±8%) to MITF. Representative images of cells immunolabeled for PAX6 and the same cells counterstained with DAPI are shown in **Figure 3(D)**.

## 3.4. Mature hPSC-RPE Cells Possessed RPE Morphology and Expressed RPE-Specific Genes and Proteins

After selective plating of pigmented areas to adherent cultures on collagen IV, pigmentation and RPE-like cell morphology were initially lost. Cells acquired fibroblast-like morphology and proliferated to confluence, after which cobblestone morphology and pigmentation began to reappear within two weeks of culture. Mature cells were pigmented and possessed regular hexagonal arrangement typical to RPE (**Figure 4(A)**).

Expression of RPE-specific markers was studied at the protein level with immunofluorescence. Cells co-expressed MITF in the nuclei and CRALBP in the cytoplasm and cell membranes (**Figure 4(B)**). Moreover, expression of Bestrophin (**Figure 4(C)**) and tight junction protein ZO-1 (**Figure 4(D)**) confirm the maturity of hESC-derived RPE cells. The maturated hPSC-RPE cells were analysed for RPE-specific gene expression with RT-PCR. Cells in all three test media were shown to express RPE precursor genes *MITF* and *OTX*2, as well as genes specific to mature RPE, namely *RPE*65, *BEST*1, *PMEL*17, *PEDF* and *TYR*, confirming differentiation to RPE fate. The maturated hESC-RPE (**Figure 4(E)**) and hiPSC-RPE (data not shown) cells showed identical gene expression profile.

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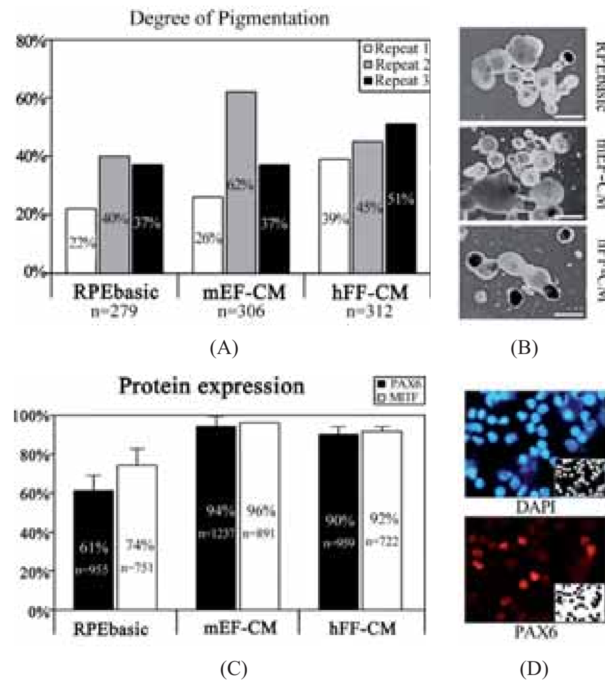


**Figure 3.** Degree of RPE differentiation at the 28 day time-point. The ratio of pigmented cell aggregates to total number of aggregates in each medium shown for three replicate experiments, n = total number of counted cell aggregates (A). Representative images of pigmented cell aggregates in each medium, scale bars 500 µm (B). Average percentage of cells expressing PAX6 and MITF in two replicate experiments quantified by cell counting. Standard deviations as error bars, n = total number of counted cells (C). Illustrative images of cells labelled with anti-PAX6 for cell counting before and after thresholding (D).

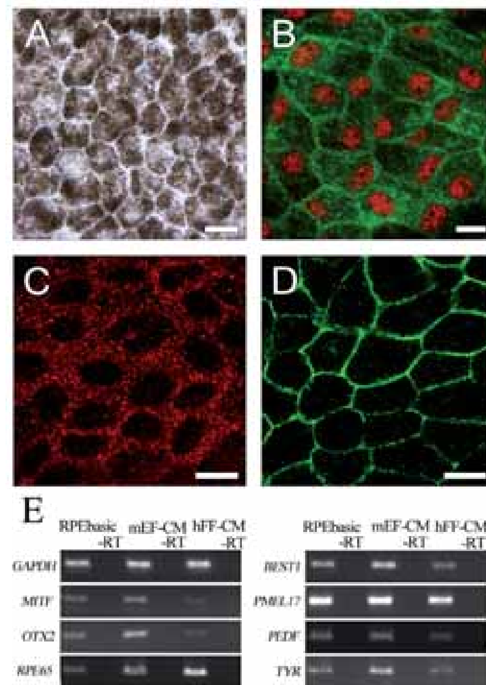


**Figure 4.** Analysis of mature hESC-RPE cells. Maturated cells possessed appropriate RPE morphology and pigmentation (A). Protein expression of CRALBP (green) and MITF (red) (B), Bestrophin (C), and ZO-1 (D) was confirmed with immunofluorescence, Scale bars 10 µm. Similar results were obtained in each test medium. Representative images in (A)-(D) are of cells cultured in mEF-CM. Gene expression profile of several RPE-related genes shown for hESC-RPE differentiated in the three test media, -RT = genomic control (E).

## 3.5. Feeder Cells Secreted Activin A and TGF-$\beta$1

Concentrations of bFGF, activin A and TGF-$\beta$1 were measured in both CM and RPEbasic with ELISA. Concentration of bFGF was undetected in all tested media. Concentration of TGF$\beta$ in RPEbasic (15% KO-SR) was 67 pg/ml. Both fibroblast types secreted low levels of TGF-$\beta$: mEF-CM contained 207 pg/ml and hFF-CM 549 pg/ml. In addition, mEFs secreted substantially more activin A compared to hFFs—mEF-CM contained 7.1 ng/ml of activin A, whereas hFF-CM contained 1.0 ng/ml. Activin A was undetected in RPEbasic, meaning that practically all the activin A present in CM was secreted by the fibroblasts.

## 3.6. Activin A Supplementation Accelerated hESC-RPE Differentiation

Based on the results of the growth factor analyses, in ductive effect of activin A was tested by supplementing RPEbasic with 10 ng/ml of recombinant human activin A. In all three separate repeats, addition of activin A had a pronounced effect on the early-stage RPE differentiation. Activin A accelerated the onset of pigmentation from an average of day 16 to day 11 (**Figure 5(A)**), and by day 28 of differentiation enhanced the degree of pigmentation from 30% to 70% of pigmented cell aggregates (**Figure 5(B)**). Furthermore, differentiation cultures treated with activin A showed higher expression of PAX6 and MITF, quantified from immunofluorescence samples. On aver- age, 96% (± 1%) of cells were positive for PAX6 and 71% (± 14%) for MITF after activin A treatment, while corresponding values in RPEbasic were 74% (± 16%) and 57% (± 13%) (**Figure 5C**). After adherent maturation culture, the cells in RPEbasic supplemented with 10 ng/ml activin A showed mature RPE phenotype with corresponding pigmentation, morphology and protein expression (**Figure 5 (D)-(G)**).

## 4. DISCUSSION

During early eye development, RPE is surrounded by the

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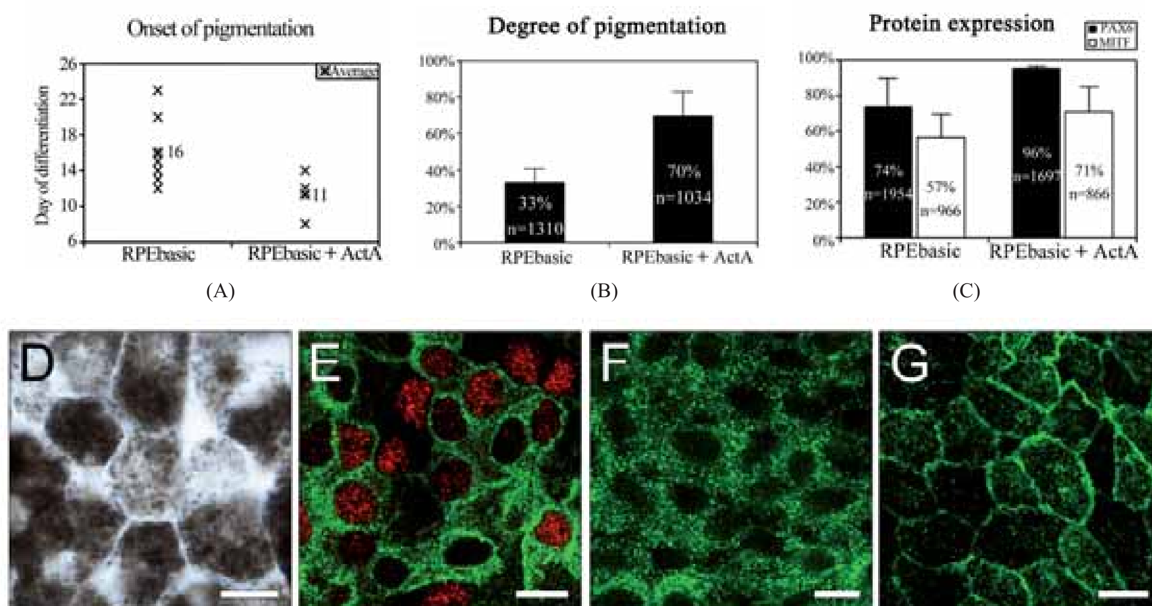


**Figure 5.** Differentiation efficacy of medium supplemented with activin A. Addition of activin A to the RPEbasic medium had a positive effect on the onset of pigmentation (A). Similarly, ratio of pigmented cell aggregates after 28 days of differentiation was enhanced, n = total number of cell aggregates counted (B). Percentage of PAX6 and MITF positive cells with and without activin A supplementation (C). Mature hESC-RPE cells cultured in RPEbasic supplemented with activin A possessed appropriate cell morphology and pigmentation (D) and expressed CRALBP (green) and MITF (red) (E), Bestrophin (F), and ZO-1 (G). Scale bars 10 µm.

extraocular mesenchyme, while the ectoderm faces the neural retina. RPE cell differentiation is known to be regulated by two key regulatory transcription factors *MITF* and *OTX*2. Expression of these transcription factors is controlled by interactions with the surrounding extraocular tissue, including the extraocular mesenchyime [12].

In the present study we hypothesized that fibroblast feeder cells used for the culture of undifferentiated hPSC may provide variable mesechymal signals having an inductive effect on spontaneous RPE cell differentiation *in vitro*. The results of this study clearly demonstrated the inductive effect of the two most commonly used fibroblast feeder cell types, mEFs and hFFs, on RPE cell differentiation both from hESC and iPSCs. In the presence of soluble factors secreted by feeder cells, both the onset of pigmentation and its rate were clearly enhanced. As expected, there was considerable biological variation in the appearance and amount of pigmentation between the replicate experiments typical for suspension culture methods. However, a clear correlating trend was observed. Along with the appearance of pigmented cells, eye field transcription factor genes *RAX* and *PAX6* were expressed. After four weeks of differentiation, expression of RPE-specific transcription factor *MITF* was the highest in cells differentiated in mEF-CM, accompanied by decreased expression of *RAX* and a low expression of *CRX* and *CHX*10 demonstrating the early neural precursors' progress towards RPE cell fate instead of neural retina. Similar but moderated effect was seen with cells differentiated in hFF-CM. Most importantly, both of the CM conditions were verified to contain substantially more PAX6 and MITF expressing cells compared to non-conditioned RPEbasic at the protein level, using quantitative cell counting. After selective plating of pigmented clusters to adherent culture, the cells showed mature RPE morphology and expression of RPE-specific markers, both at gene and protein level. Taken together, the induction of RPE differentiation with feeder cell CM had a positive effect on hPSC-RPE differentiation.

Fibroblast feeder cells in general are known to secrete various factors promoting or inhibiting the growth and differentiation of hPSC cells [33-37]. To elucidate the inductive effect of CM in RPE differentiation, we further studied the secretion of bFGF, TGF-$\beta$1 and activin A, known factors regulating eye field differentiation, by the feeder cells. As a result we found that secretion of activin A was substantially higher by mEFs compared to hFFs. In contrast, secretion of TGF-$\beta$ was higher for hFFs compared to mEFs. This is consistent with our previous studies showing that mEFs secrete more activin A and hFFs secrete more TGF$\beta$ [38]. We were not able to detect any measurable levels of bFGF from either CM thus possible effect of difference in bFGF concentration was excluded. Similar trend in fibroblast growth factor secre-

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tion has been confirmed by another research group [32]. The extraocular mesenchyme secretes TGF-$\beta$1 superfamily growth factors such as activin A, activates the expression of *MITF* and down-regulates *CHX10* expression directing RPE cell fate differentiation *in vivo*. Similar effects of activin A inducing *MITF* expression have been shown [26]. Activin A has also been shown to induce hESC-RPE differentiation *in vitro,* but only after pre- treatment with nicotinamide [17,39]. The superior secretion of activin A by mEF feeder cells could thus be one of the key factors enhancing the early RPE differentiation and reduction of the *RAX*, *CRX*, *CHX*10 expression.

To study the effect of activin A secretion by mEF, we supplemented the RPEbasic medium with 10 ng/ml activin A and concluded that addition of activin A at this low level had a pronounced effect on the early-stage RPE differentiation. In previously published studies, relatively high activin A concentrations of 140 ng/ml between day 14-28 of differentiation [17] and 100 ng/ml between day 20-40 [40] have been used. On the contrary, we were able to induce early RPE differentiation with substantially lower activin A concentration. However, in addition to activin A both mEF-CM and hFF-CM may contain a pool of other possible factors inducing RPE cell differentiation. Both fibroblast types secrete various ECM components like collagens I and IV, nidogen I, and fibronectin as well as proteins involved in TGF$\beta$, BMP, Wnt and IGF signaling [33]. In addition mEFs secrete the neurotrophic pigment epithelium derived factor (PEDF) [33,34] leaving the field open to identify other important players.

## 5. CONCLUSION

In this study, we confirmed the inductive effect of commonly used fibroblast feeder cells on hPSC differentiation towards RPE cells. Human PSCs were differentiated using media conditioned by two types of fibroblasts originated from mouse embryos and neonatal human foreskin tissue. Both feeder cell type CM increased RPE differentiation as compared to the non-conditioned medium (RPEbasic). The growth factor activin A, known inductive agent of RPE fate, was concluded to be an important factor present especially in mEF-CM. Consequently, supplementation of RPEbasic medium with a low concentration of activin A increased the differentiation rate of RPE cells to comparative level achieved with CM. Thus, inductive effect provided by feeder cells was at least partially driven by activin A.

## 6. ACKNOWLEDGEMENTS

We thank Professor Timo Otonkoski's group at University of Helsinki for the kind gift of the hiPSC line FiPS5-7. We thank Outi Melin, Hanna Koskenaho and Elina Konsén for technical assistance. The study was financially supported by Academy of Finland (218050; 133879), The Competitive Research Funding of the Tampere University Hospital (9H114; 9M098), Päivikki and Sakari Sohlberg foundation, Finnish Cultural Foundation and Tampere Graduate Program in Biomedicine and Biotechnology. The PAX6 antibody developed by Kawakami A was obtained from the Developmental Studies Hybridoma Bank (DHSB) developed under auspices of the NICHD and maintained by the University of IOWA, Department of Biology, Iowa City, IA, 52242.

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