KRISTIINA RAJALA

Development of Human Stem Cell Culture Conditions for Clinical Cell Therapy

ACADEMIC DISSERTATION
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To my family

Abstract

The hallmark of all undifferentiated stem cells is their nearly unlimited self-renewal capacity and their potential to differentiate into a diverse range of specialised cell types. These unique properties of stem cells make them invaluable research tools and can potentially serve as a source of cells for regenerative therapies. Pluripotent stem cells such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPS cells) are capable of producing almost any cell type in the human body, whereas multipotent adult stem cells exhibit limited self-renewal and a differentiation capacity that is restricted to the cell types of a particular lineage or a closely related family of cells. Since the discovery of stem cells, diverse culture conditions have been evaluated for different types of stem cells. In current standard *in vitro* cell culture techniques, xenogeneic reagents are used in the establishment, cultivation, and differentiation procedures. For the clinical applications of stem cells, however, xenogeneic reagents pose the risk of a severe immune response, and the transmission of viral or bacterial infections, prions, and unidentified zoonoses.

In this study, we assessed the applicability of an automated cell culturing, imaging, and analysis system to evaluate undifferentiated growth dynamics of hESCs maintained in different culture media. The molecular mechanisms regulating self-renewal and pluripotency of hESCs under hypoxic conditions were also elucidated. Furthermore, defined and xeno-free culture conditions for the expansion of stem cells were evaluated, developed, and optimised to meet the regulatory standards set by the directives of the European Union for the clinical application of stem cells. The applicability of a defined xeno-free medium for the derivation and maintenance of hESCs as well as for the expansion of iPS cells and adipose stem cells (ASCs) was evaluated.

The results indicated that the automated cell culturing, imaging, and analysis system enables reliable analysis of the undifferentiated growth dynamics of hESCs in different culture conditions, and revealed more information than does conventional microscopic observation. Exposure to hypoxic conditions prevented spontaneous differentiation, supported self-renewal, and significantly increased the hESC proliferation. Fundamental differences in genes that are central to hypoxia signalling, calcium and PKC pathway, and the retinoic acid pathway were detected in different culture conditions under hypoxia. A key transcription factor for self-renewal, Oct-4, was significantly upregulated under hypoxic conditions, indicating a possible mechanism for hypoxia-induced self-renewal and prevention of spontaneous differentiation.

The results of these studies suggest that although a population of hESCs was able to adapt to human serum-containing culture conditions, several of the xeno-free culture media evaluated were unable to maintain hESC self-renewal. Here we developed a xeno-free medium formulation, RegES, that allowed for the derivation of

pluripotent hESC lines. Furthermore, hESCs, iPS cells, and ASCs can be propagated in the RegES medium for a prolonged period of time with a reasonable proliferation rate while maintaining their characteristics and differentiation potential.

Clinical stem cell therapy trials are ongoing, which calls for a strong focus on the safety and quality of *in vitro* expanded stem cell transplants. By replacing xenogeneic products with a defined xeno-free medium, the safety and quality of the cells with therapeutic potential may be enhanced significantly. The use of completely defined conditions will allow for a better understanding of stem cell regulation and differentiation as well as provide more reproducible and reliable results. Culture conditions may have significant impact on the cell characteristics, thus proper characterization of cells with specific analyzing methods is necessary. Future studies should focus on validating the xeno-free culture conditions to demonstrate the ability for long-term culture, maintenance of key features of self-renewal, differentiation potency, and genetic stability, as well as derivation, reprogramming, or isolation of new stem cell lines as a full proof-of-principle, and to provide for scale-up to a manufacturing level. Additional pre-clinical safety and efficacy studies are needed before the promise of the xeno-free products can be fully realised. The results of the present study indicate that the xeno-free RegES medium is applicable for further optimization of xeno-free establishment, culture, and differentiation of various stem cell types and can ultimately serve as a platform for the production of clinical-grade multi- and pluripotent stem cells and their derivatives for safer clinical cell-based therapy.

Tiivistelmä

Erilaistumattomien kantasolujen erityispiirteitä ovat lähes rajaton jakautumiskyky sekä kyky erilaistua moniksi erilaistuneiksi solutyypeiksi. Näiden erityispiirteiden ansiosta kantasoluja voidaan hyödyntää sekä tutkimuksessa että regeneratiivisessa lääketieteessä. Pluripotentit kantasolut, kuten ihmisen alkion kantasolut ja indusoidut pluripotentit kantasolut, pystyvät erilaistumaan ainakin teoriassa miksi tahansa aikuisen yksilön solutyypiksi kun taas monikykyisillä aikuisen kantasoluilla on rajallinen jakaantumis- ja erilaistumiskyky, jolloin ne voivat muodostaa erilaisia solutyyppejä tietyn solulinjan sisällä. Aikojen kuluessa kantasoluja on viljelty monissa erilaisissa olosuhteissa. Tällä hetkellä yleisesti käytössä olevissa kantasolujen johtamis-, viljely-, ja erilaistamistekniikoissa käytetään eläinperäisiä ainesosia. Kliinisessä käytössä eläinperäiset ainesosat voivat aiheuttaa vakavan immunivasteen, viruksien ja bakteerien aiheuttamia infektioita, prionitauteja sekä toistaiseksi tunnistamattomia eläintauteja.

Tässä työssä tutkittiin automatisoidun viljely-, kuvantamis- ja analyysimenetelmän soveltuvuutta erilaisissa viljelyolosuhteissa kasvatettujen erilaistumattomien ihmisen alkion kantasolujen kasvudynamiikan evaluoimiseksi. Lisäksi työssä tutkittiin alhaisen happipitoisuuden vaikutuksia ihmisen alkion kantasolujen uusiutumis- ja erilaistumiskykyyn sekä niihin vaikuttavia molekyylitason mekanismeja. Edelleen koostumukseltaan tunnettuja ja eläinperäisiä ainesosia sisältämättömiä kantasolujen viljelyolosuhteita evaluoitiin, kehitettiin ja optimoitiin täyttämään Euroopan Unionin direktiivien asettamat viranomaisvaatimukset kantasolujen kliinisistä sovelluksista. Työssä evaluoitiin koostumukseltaan tunnetun, eläinperäisiä ainesosia sisältämättömän viljelyliuoksen soveltuvuutta ihmisen alkion kantasolujen perustamisessa ja viljelyssä sekä indusoitujen kantasolujen ja aikuisen kantasolujen viljelyssä.

Tämän työn tulokset osoittavat, että automatisoitu viljely-, kuvantamis- ja analysointimenetelmä mahdollisti erilaisissa viljelyolosuhteissa kasvatettujen erilaistumattomien ihmisen alkion kantasolujen kasvudynamiikan analysoinnin luotettavasti, tuottaen enemmän informaatiota kuin perinteinen mikroskooppinen tarkastelu. Alhainen happipitoisuus esti ihmisen alkion kantasolujen spontaania erilaistumista, tuki uusiutumiskykyä ja lisäsi merkittävästi jakaantumista. Alhaisessa happipitoisuudessa olennaisia eroja esiintyi alhaisen happipitoisuuden, kalsium ja PKC ja retinolihapon signaalireiteille keskeisten geenien ilmentymisessä erilaisissa viljelyolosuhteissa. Yhtä uusiutumiskyvylle merkittävää transkriptiotekijää Oct-4:sta ilmentyi merkittävästi enemmän alhaisessa happipitoisuudessa, osoittaen mahdollisen mekanismin alhaisen happipitoisuuden indusoimalle uusiutumiskyvyn lisääntymiselle ja spontaanin erilaistumisen estämiselle.

Tämän työn tulokset osoittavat, että useat eläinperäisiä ainesosia sisältävät viljelyliuokset eivät pystyneet ylläpitämään ihmisen alkion kantasolujen

erilaistumatonta kasvua. Osa ihmisen alkion kantasoluista kuitenkin kykeni adaptoitumaan ihmisen seerumia sisältävään viljelyolosuhteeseen. Tässä työssä kehitetty koostumukseltaan tunnettu ja eläinperäisiä ainesosia sisältämätön viljelyliuos RegES mahdollisti ihmisen alkion kantasolulinjojen perustamisen. Lisäksi RegES viljelyliuos mahdollisti ihmisen alkion, indusoitujen ja aikuisen kantasolujen pitkäaikaisen viljelyn, kantasolujen tehokkaan jakautumisnopeuden sekä ylläpiti kantasolujen erityispiirteitä ja erilaistumiskykyä.

Parhaillaan kantasoluja hyödyntäviä kliinisiä soluterapiakokeita on jo käynnissä, mikä edellyttää keskittymistä erityisesti laboratorio-olosuhteissa lisättyjen solusiirteiden turvallisuuteen ja laatuun. Käyttämällä koostumukseltaan tunnettuja eläinperäisiä ainesosia sisältämättömiä tuotteita voidaan solutuotteiden turvallisuutta ja laatua parantaa merkittävästi. Koostumukseltaan tunnettujen olosuhteiden käyttö mahdollistaa kantasolujen säätelyn ja erilaistumisen syvempää ymmärtämistä sekä lisää tulosten toistettavuutta ja luotettavuutta. Viljelyolosuhteilla voi olla huomattavia vaikutuksia solujen erityispiirteisiin, jonka vuoksi solujen asianmukainen karakterisointi tarkoin määritellyillä analyysimenetelmillä on ensiarvoisen tärkeää. Tulevaisuudessa pitäisi keskittyä eläinperäisiä ainesosia sisältämättömien viljelyolosuhteiden validoimiseen ja osoittaa, että ne mahdollistavat solujen pitkäaikaisen viljelyn, ylläpitävät uusiutumiskyvyn erityispiirteitä, erilaistumiskykyä ja geneettistä stabiiliutta, mahdollistavat uusien kantasolulinjojen perustamisen, uudelleenohjelmoinnin ja eristämisen, sekä tuotannon lisäyksen. Täydentäviä prekliinisiä turvallisuus- ja tehokkuustutkimuksia tarvitaan vielä ennen kuin eläinperäisiä ainesosia sisältämättömien tuotteiden kaikki hyödyntämismahdollisuudet voidaan toteuttaa. Tämä työ osoittaa, että eläinperäisiä ainesosia sisältämätön viljelyliuos RegES soveltuu erilaisten kantasolujen perustamisen, viljelyn ja erilaistamisen jatkokehitykseen ja voi tulevaisuudessa toimia perustana kliinisten monikykyisten ja pluripotenttien kantasolujen sekä näiden johdannaisten turvallisessa tuotannossa kliinisiä soluterapiahoitoja varten.

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List of abbreviations

AFP	Alfa-fetoprotein
alloHS	Allogeneic human serum
ALP	Alkaline phosphatase
APC	Allophycocyanin
ApoB-100	Apolipoprotein B-100
ASC	Adipose stem cell
ATMP	Advanced therapy medicinal product
autoHS	Autologous human serum
BAFF	B cell activating factor
BDNF	Brain-derived neurotrophic factor
BIO	Glycogen synthase kinase-2 inhibitor
BMP	Bone morphogenetic protein
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLA	Conjugated linoleic acid
CM	Conditioned medium
c-Myc	V-myc myelocytomatosis viral oncogene homolog (avian)
CREB	cAMP response element-binding
CSF2	Colony stimulating factor 2
Ct	Cycle threshold
CTAD	C-terminal activation domain
DAPI	4,6-diamidino-2-phenylindole
DCTN2	Dynactin subunit 2
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	Dulbecco's modified Eagle's medium: nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
EB	Embryoid body
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicinal Agency
END-2	Mouse visceral endodermal-like cells
EPA	Eicosapentaenoic acid
ErbB	Human epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FDA	Unites States Food and Drug Administration
FGF	Fibroblast growth factor (b, basic)

FITC	Fluorescein isothiocyanate isomer 1
FL	Flt3 ligand, Fms-related tyrosine kinase 3 ligand
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
GABRB3	Gamma-aminobutyric acid receptor subunit beta-3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF3	Growth differentiation factor-3
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GMP	Good manufacturing practice (c, current)
Gremlin-1	Cysteine knot superfamily, homolog (Xenopus laevis)
GTP	Good tissue practice
hDF	Human dermal fibroblast
hEL-CM	Human embryonic lung fibroblast- conditioned medium
hESC	Human embryonic stem cell
hESC-df	Human embryonic stem cell-derived fibroblast
hFF	Human foreskin fibroblast
HIF	Hypoxia inducible factor
HLA-ABC	Human leukocyte antigen class I
HLA-DR	Human leukocyte antigen class II
HNF3B	Hepatocyte nuclear factor
HRE	Hypoxia-response element
HRG-β1	Heregulin-beta1
HSA	Human serum albumin
ICM	Inner cell mass
IGF	Insulin-like growth factor
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IND	Investigational new drug
iPS cell	Induced pluripotent stem cell
ISSCR	International Society for Stem Cell Research
ITS+1	Insulin, transferrin, selenous acid, bovine serum albumin, linoleic acid
IVF	In vitro fertilization
Jak	Janus kinase
KGF	Keratinocyte growth factor
Klf	Krupper-like family of transcription factors
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)
LPA	Lysophosphatidic acid
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MHC I, II	Major histocompatibility complex class I and II
MSC	Mesenchymal stem/stromal cell
Musashi	Musashi homolog 1 (Drosophila)
Myc	Myc family of transcription factors
Nanog	Nanog homeobox

NCAM	Neural cell adhesion molecule
NC-CM	Neonatal chondrocyte-conditioned medium
NEAA	Non-essential amino acids
Neu5Gc	N-glycoylneuraminic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-68	Neurofilament 68
Nodal	Nodal homolog
Nr5a2	Nuclear receptor subfamily 5, group A, member 2
NT	Neurotrophin
Oct-4	Octamer-4, POU domain, class 5, transcription factor 1
Olig1	Oligodendrocyte transcription factor 1
OTX2	Orthodenticle homeobox 2
PAX-6	Paired box gene 6
PBS	Phosphate buffered saline (d, Dulbecco's)
PCR	Polymerase chain reaction (RT, reverse transcription; qRT, quantitative real-time)
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PRKCE	Protein kinase C epsilon type
PTN	Pleiotrophin
Rex-1	Zinc finger protein 42 homolog
ROCK	Rho-associated kinase inhibitor
SCED	Single-cell enzymatic passaging
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SCNT	Somatic cell nuclear transfer
SFM	Serum-free medium
SMAD	Transforming growth factor beta ligand
SOX	Sex determining region Y-box
S1P	Sphingosine-1-phosphate
Src	Sarcoma family of proto-oncogenic tyrosine kinases
SSEA	Stage-specific embryonic antigen
STAT	Signal Transducers and Activator of Transcription
TDGF1	Teratocarcinoma-derived growth factor 1
TFRC	Transferrin receptor
TGF-β	Transforming growth factor beta
Thy-1	Thy-1 cell surface antigen CD90
TRA	Tumor-related antigen, keratan sulfate-related antigen
VEGFA	Vascular endothelial growth factor A
vMHC	Ventricular myosin heavy chain
WNT	Wingless-type MMTV integration site family

List of original publications

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

I. Narkilahti S*, **Rajala K***, Pihlajamäki H, Suuronen R, Hovatta O, Skottman H. Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods. Biomed Eng Online 2007, 6(11). * equal contribution

II. **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 culture. Hum Reprod 2007, 22(5):1231-1238.

III. **Rajala K**, Vaajasaari H, Suuronen R, Hovatta O, Skottman H. Effects of the physiochemical culture environment on the stemness and pluripotency of human embryonic stem cells. *Submitted.*

IV. **Rajala K**, Lindroos B, Hussein SM, Lappalainen RS, Pekkanen-Mattila M, Inzunza J, Rozell B, Miettinen S, Narkilahti S, Kerkelä E, Aalto-Setälä K, Otonkoski T, Suuronen R, Hovatta O, Skottman H. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. Plos One 2010, 5(4).

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1. Introduction

The goal of regenerative medicine is to use cell-based therapy, biomaterials alone, or a combination of cells and biomaterials to replace and repair cells, tissues, or organs that are damaged due to disease or injury (Atala, 2006; Zuk, 2008). Therapeutic application of stem cells and their further differentiated progenitors is a promising and rapidly emerging area of regenerative medicine in which stem cell-based treatments could be applied to treat numerous genetic and degenerative disorders. The general strategy for stem cell therapies is the expansion of undifferentiated stem cells, followed by differentiation to a specific cell type and delivery to the patient, where the cells functionally integrate into the damaged tissue and restore its normal function (Carpenter et al., 2009). Genetically modified stem cells could even be used to reverse inherited genetic defects that are responsible for diverse pathological disorders in humans and as vehicles to specifically deliver the therapeutic molecules in damaged tissues or organs (Strulovici et al., 2007; Choumerianou et al., 2008). Furthermore, *in vivo* stimulation of endogenous adult stem cells using specific growth factors leads to the development of novel stem cell-based therapeutic approaches for regenerative medicine (Xu et al., 2008). Besides showing enormous potential for transplantation therapies, intense research on stem cells during the last few decades has revealed that these cells are invaluable tools for studying the early events of development, stem cell biology in general, as well as basic disease mechanisms, and can be used as an ideal biological platform for drug discovery and testing.

Stem cells are characterised as undifferentiated cells capable of self-renewal and differentiation into a diverse range of specialised cell types. According to their origin, human stem cells are classified as adult, fetal, embryonic (ESCs), and induced pluripotent stem cells (iPS cells). Adult and fetal stem cells reside in adult and fetal tissues and ESCs can be isolated from the inner cells mass (ICM) of blastocysts, whereas iPS cells are artificially derived from a somatic cell, by inducing the "forced" expression of certain genes. The developing organs and tissues in a fetus contain stem cells that are needed for growth and maturation while the primary roles of adult stem cells in the human body are to repopulate the tissues by generating new mature cell types and regenerating damaged tissue in response to injury or disease. The ESCs and iPS cells exhibit nearly unlimited self-renewal capacity and are able to differentiate into a wide range of cell types, whereas tissue-specific adult stem cells have limited self-renewal and differentiation capacity giving rise to only specific cell types (Choumerianou et al., 2008; Hipp and Atala, 2008).

The major advantage of iPS and adult stem cells is that they can be used in patient-specific autologous therapies, thus avoiding immune rejection complications, whereas the use of ESCs may result in adverse immune reactions (Mimeault and Batra, 2006; Moore and Lemischka, 2006; Mimeault et al., 2007; Grinnemo et al.,

2008). Adult stem cells and iPS cells, however, are likely produced only after initiation of the disease or damage in a patient, precluding their use in the acute phase of the injury. Diseases that might benefit from stem cell-based therapies include age-related functional defects, diabetes, heart disease, bone or connective tissue disorders, haematological and immune system disorders, cerebrovascular disease, liver and renal failure, spinal cord injuries, Alzheimer's diseases and Parkinson's disease, as well as many aggressive and recurrent cancers (Choumerianou et al., 2008; Hipp and Atala, 2008; Mountford, 2008).

Although cell therapies using adult stem cells for several disorders have been in use for many decades since 1968, beginning with the first successful bone marrow transplant (Gatti et al., 1968; Bach and Boitard 1986), stem cell therapy has many hurdles to overcome before it will become a viable and widely used clinical option. To fully achieve the promise of regenerative medicine, it is necessary to understand the biology and properties of stem cells, to achieve efficient *in vitro* expansion and differentiation of stem cells to completely functional cell types, and to overcome the post-transplantation challenges that limit their use, such as tumor risk, genetic instability, and immune rejection.

2. Review of literature

2.1 Stem cells

2.1.1 Classification and sources of stem cells

The common hallmark of all the undifferentiated stem cells is their nearly unlimited self-renewal capacity and differentiation potential that confer their primordial and vital role during the developmental process and throughout the lifespan in adult mammals. These unique properties make stem cells invaluable tools for studying stem cell biology and various diseases and show promise in providing a new source of cells for transplantation therapies and *in vitro* models for pharmaceutical testing.

In mammalian development, the fertilised egg, the zygote, has the ability to generate an entire organism comprising more than 200 different cell types. During development, stem cells divide and produce more specialised cells. The zygote, with its ability to produce all of the differentiated cell types, including the extra embryonic tissues, is **totipotent**. Cell differentiation subsequently results in the formation of a blastocyst composed of outer cells and inner cells, known as the ICM, which remains undifferentiated. The outer cells form trophoblast cells that later develop into the embryonic membranes and placenta. The cells of the ICM are no longer totipotent, but retain the ability to develop into all cell types of the embryo proper, i.e., they remain **pluripotent** (Wobus and Boheler, 2005). During embryogenesis, the pluripotent stem cells of the ICM generate the primitive ectoderm that eventually gives rise to the three primary germ layers: ectoderm, mesoderm, and endoderm. The cells of each primary germ layer are committed to generating cells from multiple, but still a limited number, of lineages, i.e., they are **multipotent**. The ectoderm gives rise to the nervous system and the epidermis. The mesoderm gives rise to the connective tissue, muscles, bones, blood, and most of the internal organs, whereas the endoderm forms the gastrointestinal tract (Wobus and Boheler, 2005; Choumerianou et al., 2008). Throughout the life of an organism, multipotent stem and progenitor cells are present in various tissues and organs, regenerating and repairing the tissue in response to injury or disease.

Human stem cells are classified according to their origin and their differentiation potential (Figure 1). Depending on their origin, stem cells are capable of producing any cell type of the human body, excluding extra embryonic tissues, and are referred to as pluripotent (ESCs and iPS cells), or they produce some specific cell types of a particular lineage or closely related family, and are referred to as multipotent (fetal and adult stem cells) (Choumerianou et al., 2008).

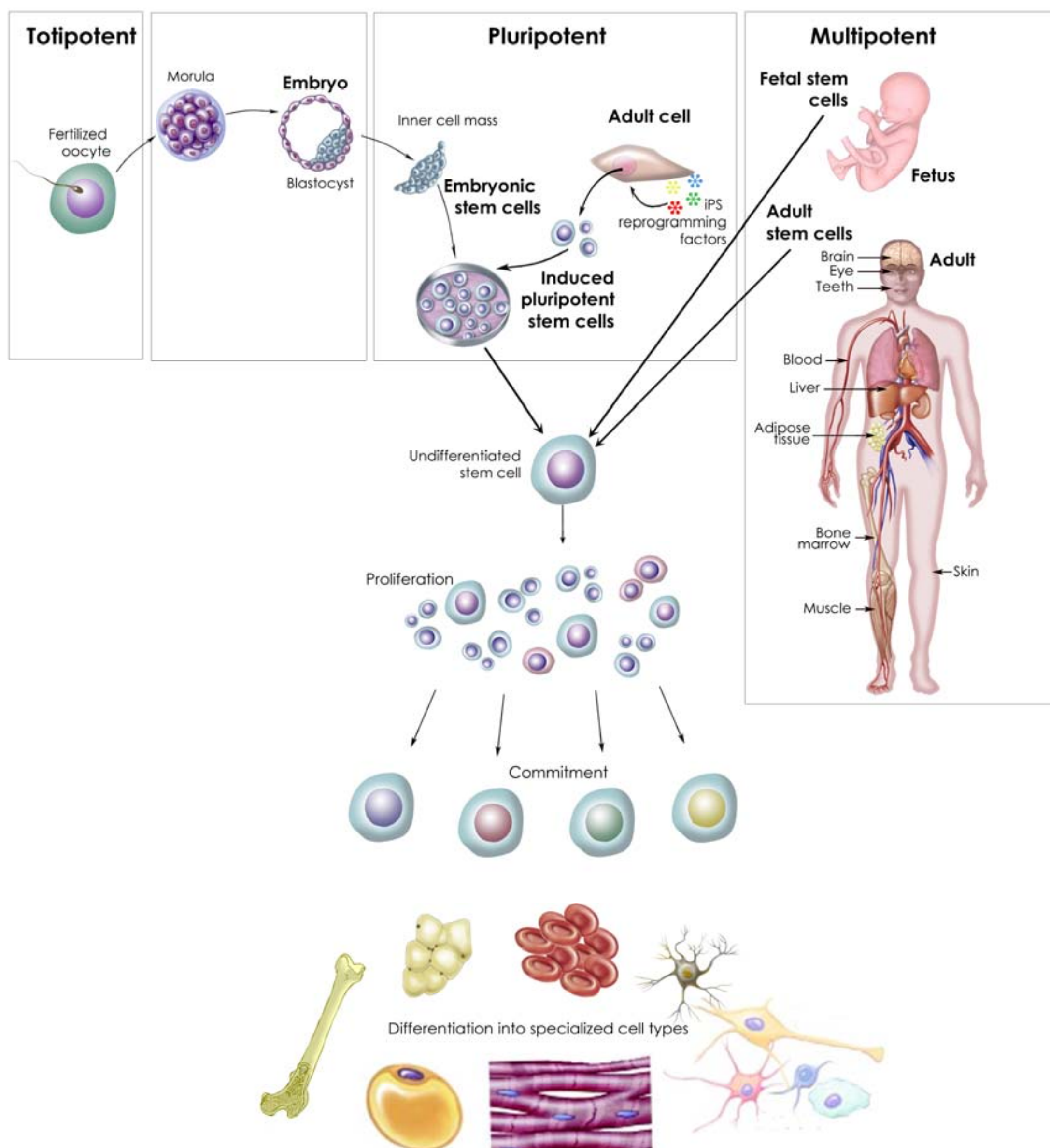

Figure 1. Stem cell hierarchy. The zygote is defined as totipotent, because it gives rise to a complex organism. At the blastocyst stage, only the cells of the ICM, ESCs derived from the ICM, and iPS cells derived from somatic cells retain the capacity to differentiate into all three primary germ layers, the endoderm, mesoderm, and ectoderm, as well as the primordial germ cells, and are defined as pluripotent. The developing organs and tissues in a fetus contain partly matured multipotent stem cells that have more restricted differentiation potential than pluripotent stem cells. Throughout adult life, multipotent stem and progenitor cells continue to reside in various tissues and organs, replacing lost or injured cells. Picture modified from image prepared by Bettina Lindroos in the thesis: *Characterization and Optimization of in vitro culture conditions of adult stem cells for clinical cell therapy.* Acta Universitatis Tamperensis 1477. Original images were prepared by Catherine Twomey for the National Academies *Understanding stem cells: An Overview of the Science and Issues,* http://www.nationalacademies.org/stemcells.

2.1.2 Fetal stem cells

Fetal stem cells can be isolated from various organs and tissues of the fetus or from supportive extra embryonic structures of fetal origin (Hemberger et al., 2008). Fetal stem cells have been isolated from fetal tissues, including bone marrow, liver, blood, lung, spleen, pancreas and kidney, and from several extra embryonic tissues, including umbilical cord blood, amniotic fluid and membrane, Wharton's jelly, and placenta (Campagnoli et al., 2001; In 't Anker et al., 2003; In 't Anker et al., 2004; Pappa and Anagnou, 2009). Fetal stem cells exhibit several features of ESCs, such as the expression of stem cell markers and their ability to self-renew (Guillot et al., 2007). The differentiation potential of some specific types of fetal stem cells recapitulates features of plasticity residing between pluripotent and multipotent stem cells, while other types of fetal stem cells are merely multipotent (Pappa and Anagnou, 2009). Fetal stem cells can differentiate into functional haematopoietic cells, adipocytes, chondrocytes, osteocytes, cardiomyocytes, hepatocytes, insulin-secreting β-cells, lung progenitor cells, muscle cells, and neural cells, including dopaminergic neurons and glia (Mimeault et al., 2007).

To date, studies of fetal stem cells have provided important information and new insights into the understanding of the biology of stem cells in general and have suggested strategies to utilise their therapeutic potential. Several problems, however, are associated with the therapeutic use of prenatal fetal stem cells. The stem cells in fetal tissues are present in low numbers and need to be greatly expanded *in vitro* to be sufficient for the therapeutic needs of adults. In addition, tissue rejection may limit the usefulness of fetal stem cells for human clinical applications. Stem cells from extra embryonic sources, mostly of the mesenchymal type, are a particularly interesting source of stem cells for regenerative medicine because they show an expansion potential superior to that of stem cells isolated from adult tissues, demonstrate no teratoma formation, and appear to be less immunogenic (In 't Anker et al., 2003; Gang et al., 2004; Pappa and Anagnou, 2009).

2.1.3 Adult stem cells

Adult stem cells exhibit a limited self-renewal and differentiation capacity that is restricted to the cell types of a particular lineage or a closely related family of cells (Choumerianou et a., 2008; Hipp and Atala 2008). The major advantage of adult stem cells is that they can be used in autologous therapies, thus avoiding any immune rejection complications (Mimeault and Batra, 2006; Moore and Lemischka, 2006; Mimeault et al., 2007). It has been known for decades that bone marrow contains two types of stem cells: haematopoietic stem cells, which are committed to differentiate into all the haematopoietic cell lineages in blood, and the less-differentiated stromal mesenchymal cells (Choumerianou et al., 2008). Within the last decade, however, adult stem cells have been identified in other organs and tissues, including brain, peripheral blood, blood vessels, skeletal muscle, skin, adipose tissue, dental pulp, liver, pancreas, eyes, kidneys, lungs, heart, gut, liver, ovarian epithelium, prostate, and testis (Presnell et al., 2002; Jiang et al., 2002; Mimeault et al., 2007). Besides haematopoietic stem cells, the most characterised and widely-used type of adult stem cells are the mesenchymal stem cells (MSCs),

which are found in various tissues throughout the body, e.g., bone marrow, skin, fat, and muscle. MSCs, which have a broad plasticity and greater differentiation potential than many other adult stem cell types, can give rise to a large variety of specialised mesenchymal tissues including bone, cartilage, fat, muscle, tendon, ligament, and other kinds of connective tissue (Pittenger et al., 1999).

While many adult stem cells, including MSCs, are present at a low frequency (on the order of 1 in 10000 cells within the tissue), have limited capacity to divide, and are difficult to expand in culture conditions, human adipose stem cells (ASCs) are an abundant, readily available population of multipotent progenitor cells that reside in adipose tissue and can easily be expanded *in vitro* (Schaffler and Buchler, 2007; Lindroos et al., 2009). Large numbers of ASCs can be retrieved from adipose tissue and can be induced to undergo adipogenic, osteogenic, chondrogenic, neurogenic, and myogenic differentiation *in vitro* (Zuk et al., 2001; Schaffler and Buchler, 2007; Lindroos et al., 2009). ASCs are typically characterised by their immunophenotype in the undifferentiated state and by their differentiation potential towards the adipogenic, osteogenic, and chondrogenic lineages in the presence of lineage-specific growth factors and cytokines (Gimble and Guilak, 2003). Furthermore, ASCs exhibit immunoprivileged properties and lack the expression of human leukocyte antigen class II (HLA-DR), and thus demonstrate therapeutic applicability in pre-clinical studies in diverse fields (Zuk et al., 2001, Lindroos et al., 2009). While the current use of adult stem cells is quite limited, mostly due to challenges involved in isolation, maintenance, and expansion of these cells, there is great potential for the future utilization of these cells in tissue-specific regenerative therapies.

2.1.4 Human embryonic stem cells

Human ESCs are generally derived from the ICM of 5-day-old blastocysts. Human ESC lines can also be created from the whole blastocyst or earlier morula stage embryos, albeit at a lower efficiency than those from the ICM of blastocysts (Reubinoff et al., 2000; Strelchenko et al., 2004; Chen et al., 2005). The first permanent hESC line was established in 1998 from the ICM of a preimplantation embryo (Thomson et al., 1998). Since 1998, over 650 hESC lines have been registered in the EU (www.hESCreg.eu.). In most cases, excess or poor quality embryos donated for research by couples undergoing *in vitro* fertilization (IVF) treatments that would otherwise be discarded are used to create hESC lines (Lei et al., 2007; Skottman et al., 2007). The most commonly used method to derive hESC lines is isolation of the ICM using pronase and immunosurgery (Thomson et al., 1998). In immunosurgery, the blastocyst is incubated with mouse antibodies specific to human trophoectoderm and guinea pig complement proteins, resulting in lysis of the trophoectoderm so that the only surviving cells constitute the ICM (Thomson et al., 1998; Hipp and Atala, 2008). Later, other methods using Tyrode’s acid or mechanical removal of the zona pellucida instead of pronase, followed by mechanical isolation of the ICM, were developed for the derivation of hESC lines, thus avoiding contact of the blastocyst with xenogeneic (i.e., animal-derived) factors (Genbacev et al., 2005; Ström et al., 2007).

Due to ethical restrictions surrounding research with hESCs, new methods are being developed to derive hESC lines without destroying human embryos. Human ESC lines can also be derived from late (day 6-7) arrested embryos (Zhang et al., 2006) and from single blastomeres of an arrested four-cell-stage embryo (Feki et al., 2008). Arrested embryos have stopped dividing and have unequal or fragmented cells and blastomeres. These arrested embryos constitute over half of the embryos produced by IVF procedures and are usually discarded. Single cell blastomeres have also been used to derive new hESC lines without destroying the embryo (Klimanskaya et al., 2006; Eiges et al., 2007). This alternative method is based on a technique used to obtain a single cell embryo biopsy for preimplantation genetic diagnosis of genetic defects.

A unique feature of hESCs that discriminates them from other types of stem cells is their ability to proliferate in long-term cultures while maintaining their pluripotent nature. The undifferentiated stage of hESCs can be monitored based on the morphological characteristics of the cells. The basic characteristics of these cells are a high nucleus to cytoplasm ratio, prominent nucleoli, and distinct colony morphology (Carpenter et al., 2003; Draper et al., 2004). Human ESCs express high levels of telomerase, which explains their ability to undergo nearly unlimited self-renewal (Thomson et al., 1998; Reubinoff et al., 2000). In addition, hESCs are defined by alkaline phosphatase (ALP) activity, a normal karyotype, and the expression of several transcription factors and cell surface proteins (Thomson et al., 1998; Reubinoff et al., 2000). The transcription factors POU domain transcription factor Oct-4, homeobox protein Nanog, and HMG-box transcription factor Sox2 form the core regulatory network that ensures the maintenance of pluripotency, while other characteristic transcription factors expressed by hESCs include Lin-28, Rex-1, and Thy-1. The cell surface antigens most commonly used to identify hESCs are the stage-specific embryonic antigen (SSEA)-3 and SSEA-4 and the tumor-related antigen (TRA)-1-60 and TRA-1-81, as well as cluster of differentiation (CD)9 and CD24. Unlike mouse ES cells, however, hESCs do not express SSEA-1 (Thomson et al., 1998; Reubinoff et al., 2000; Richards et al., 2004; Hoffman and Carpenter, 2005a). Furthermore, hESC-specific characteristics include unique histone modification and DNA methylation patterns (Bernstein et al., 2006), specific expression of a group of microRNAs (Laurent et al., 2008), and a unique cell cycle with a shortened G1 phase (Stead et al., 2002) and pluripotency.

The pluripotent nature of hESCs allows them to be differentiated into specialised cell lineages of all three embryonic germ layers: ectoderm, endoderm, and mesoderm (Thomson et al., 1998; Reubinoff et al., 2000). The differentiation is defined by the formation of embryoid bodies (EB) *in vitro* and teratoma formation *in vivo* when transplanted into severe combined immunodeficient (SCID) mice (Thomson et al., 1998; Reubinoff et al., 2000). *In vitro*, hESCs have been directly differentiated into various different cell types, such as neurons (Reubinoff et al., 2001; Schuldiner et al., 2001; Zhang et al., 2001), oligodendrocytes (Zhang et al., 2001), dopaminergic neurons (Zhang et al., 2001), astrocytes (Zhang et al., 2001), hepatocytes (Hay et al., 2007), osteoblasts (Kärner et al., 2007), chondrocytes (Toh et al., 2007), skeletal muscle (Zheng et al., 2006), retinal cells (Haruta et al., 2005), keratinocytes (Ji et al., 2006), cardiomyocytes (Kehat et al., 2001), haematopoietic cell lineages (Chadwick et al., 2003), endothelial cells (Levenberg et al., 2002), insulin producing β-cells (Assady et al., 2001), and germ cells (Clark et al., 2004).

Human ESC lines differ from each other due divergences in isolation procedures, isolation stage (e.g., morula, epiblast) or individual allelic differences, further resulting in divergences in growth rates, differentiation capacity, karyotypic stability, and the ability to integrate and function *in vivo* (Carpenter et al., 2009).

Intense research on hESCs during the last decade indicates that these cells are invaluable tools for studying the early events of development, stem cell biology in general, as well as basic disease mechanisms, and can be used as an ideal biological platform for drug discovery and testing. Most importantly, hESCs possess an enormous developmental potential that could be utilised to treat and even cure diverse genetic and degenerative disorders in the human body and whose pathology remains incurable with the other types of available clinical treatments. There are still major challenges including technical limitations regarding the quality of hESC culture, efficient differentiation of hESCs to fully functional cell types, the risk of teratoma formation, and the potential immunogenicity of hESCs, which need to be solved before hESCs can be safely used as a source for cell therapies.

2.1.5 Human induced pluripotent stem cells

During cellular differentiation, cells become increasingly more specialised and restricted in their developmental potential. Several techniques have been developed to de-differentiate adult somatic cells to produce patient-specific pluripotent stem cells without the use of embryos. Pluripotency can be induced in somatic cells by somatic cell fusion with pluripotent stem cells (Cowan et al., 2005), trans-differentiation of male germ cells (Kanatsu-Shinohara et al., 2004), parthenogenesis (Kim et al., 2007), and somatic cell nuclear transfer (SCNT) where the oocyte nucleus is replaced with a nucleus derived from a somatic cell obtained from a donor, resulting in ESCs that are genetically identical to the donor (Wakayama et al., 2001). In theory, ESCs derived from such a blastocyst would not be rejected when transplanted into the donor. A breakthrough in nuclear transfer experiments occurred in 1997, when Wilmut and colleagues reported cloning the first mammal, the sheep named Dolly, from an adult somatic cell using SCNT (Wilmut et al., 1997). Although tremendous effort has been put into these methodologies, success with human cells is limited. So far, there are no published reports of successful derivation of cloned hESC lines by SCNT, and pluripotent cells generated by cell fusion, parthenogenesis, and *in vitro* trans-differentiation of germ cells have an abnormal karyotype and imprinting status and thus immune rejection remains an issue (Yamanaka, 2008).

A promising new source of pluripotent cells was recently discovered, as lineage-restricted human somatic cells were reprogrammed by ectopic expression of a defined set of pluripotency-related transcription factors to induce the pluripotent state. Takahashi and Yamanaka were the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts can be reprogrammed into iPS cells (Takahashi and Yamanaka, 2006). They examined 24 genes considered to be important for ESCs and identified 4 key genes that are required to bestow ESC-like properties on fibroblasts. In the first published report on human cells, Yamanaka and his colleagues used retroviral vectors, each carrying Oct-4, Sox2, c-Myc, and krupper-like family of transcription factor (Klf) 4, commonly called the Yamanaka

factors, to reprogram human dermal fibroblasts (hDFs) to iPS cells (Takahashi et al., 2007). Simultaneously in 2007, Yu, Thomson, and their colleagues demonstrated that retrovirus-mediated transfection of a different set of four transcription factors, Oct-4, Sox2, Nanog, and Lin-28, also induced pluripotency in human foreskin fibroblasts (hFFs) without introducing any oncogenes (c-Myc) (Yu et al., 2007).

Like ESCs, iPS cells have a compact colony morphology and possess immortal growth characteristics in culture. They express markers characteristic to pluripotency including Nanog, Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and ALP, and exhibit high telomerase activity. Furthermore, iPS cells seem to exhibit differentiation potential similar to that of hESCs and can differentiate *in vitro* and *in vivo* into cells of all three germ layers (Takahashi et al., 2007; Yu et al., 2007. Global genome-wide expression analysis demonstrated that DNA and histone methylation patterns are similar in human iPS and hESCs and that gene expression patterns correlate well between these two cell types, although some differences do exist and thus further characterization of iPS cell lines is required to determine the full potential of these cells (Maherali et al., 2007; Chin et al., 2009; de Souza, 2010). Human ESCs currently remain the gold standard for pluripotent cells, but the knowledge accumulated from culturing and differentiating hESCs will likely also directly apply to human iPS cells.

As somatic cells are reprogrammed to iPS cells, they shut down the genes specific for their own cell type and activate genes that maintain pluripotency. Oct-4, Sox2, Nanog, and Lin-28 contribute to the reprogramming, and c-Myc and Klf4 enhance the efficiency of clonal recovery (Park et al., 2008). What happens at the molecular level during the reprogramming process, however, is not fully understood and is the current focus in iPS cell research (Amabile and Meissner, 2009). So far, different human somatic cell types, including fibroblasts, keratinocytes, and cell types from blood have been reprogrammed to induce pluripotency (Takahashi et al., 2007; Yu et al., 2007; Aasen et al., 2008; Haase et al., 2009; Giorgetti et al., 2009). The pluripotency induction efficiency often differs between cell types, and it seems that the more differentiated the cell type, the more difficult it is to return it to the pluripotent state. In addition, in cell types with endogenously high expression levels of one or more of the factors that induce pluripotency, such as neural cells that strongly express Sox2, pluripotency may be induced with only a subset of factors (de Souza, 2010).

The initial methods used to generate iPS cells involved the use of retroviral or lentiviral vectors that integrate into the genome to deliver the factors to the cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Takahashi et al., 2007; Yu et al., 2007). Since then, however, the development in this field has been rapid and novel strategies have been applied to improve the reprogramming methods and efficiency as well as to generate iPS cells without permanent modification of the genome. Once reprogramming has occurred, endogenous counterparts of the exogenously supplied reprogramming factors are activated, indicating that exogenous factors are only required for the induction, not the maintenance of pluripotency (Takahashi et al., 2007). The nonintegrating reprogramming methods include adenoviruses, plasmid- and episomal vector-based approaches, and delivery of reprogramming factors directly as proteins (Shao and Wu, 2010). Other factors have been identified that can replace the four traditional transcription factors. Klf2

and Klf5 can replace Klf4, Sox1 and Sox3 can replace Sox2, and n-Myc and I-Myc can replace c-Myc (Nakagawa et al., 2008). Nuclear receptor subfamily 5, group A, member 2 (Nr5a2) can be used to replace Oct-4 in the reprogramming of murine somatic cells and also to enhance the reprogramming process by increasing transcription activity (Heng et al., 2010). In addition, small molecules are capable of replacing some of the reprogramming factors, e.g., the histone deacetylase inhibitor valproic acid can replace Klf4 and c-Myc for reprogramming human fibroblasts (Huangfu et al., 2008b) and other small molecules with reprogramming factors increase the efficiency of the reprogramming process and promote more complete reprogramming (Huangfu et al., 2008a; Lin et al., 2009).

The ability to return mature body cells to a pluripotent state enables the creation of patient-specific stem cell lines for the study of basic biology and various disease mechanisms, and has wide-ranging potential as a tool for drug discovery as well as for treating a number of human degenerative diseases without evoking immune rejection. To date, human iPS cells have been used for the study of the reprogramming process itself and establishment of disease-specific cell lines and the differentiation of these cell lines into the relevant cell types affected by the disease. For example, spinal motor neurons, dopaminergic neurons, and cardiomyocytes have been differentiated from iPS cell lines derived from patients suffering from a slowly progressing form of amyotrophic lateral sclerosis (Dimos et al., 2008), spinal muscular atrophy (Ebert et al., 2009), sporadic Parkinson’s disease (Soldner et al., 2009), and long QT syndrome (unpublished results). Research on iPS cells is still in its infancy, and understanding the true potential of these cells requires continued investigation and more complete comparisons to ESCs. In 2010, neuronal cells were directly induced from mouse fibroblasts by the combined expression of neural-lineage specific transcription factors (Vierbuchen et al., 2010). Whether this method will work in human somatic cells and how comparable these directly differentiated cells are to their *in vivo* counterparts will need to be investigated.

2.2 Culture of stem cells

In vivo, stem cells are generally colocalised with supporting cells within the specific regions in each tissue, which are designated as niches. A stem cell niche is a defined microenvironment in which the local signals and spatial organization of the cells generate location-dependent control over reversing cell-fate decisions, such as self-renewal and differentiation. The complex interactions via the formation of adherent junctions and the secretion of diverse soluble factors between stem cells and supportive cells contribute to the cell-fate decisions within the niches under specific physiological and pathological conditions. Arrangement of stem and supportive cells into niches organises the timing and levels of the signals that the stem cells receive, thus directing cell fate. Stem cells may also be found in clusters in the absence of a clearly defined niche of supportive cells, however, and still regulate cell fate in a spatially organised fashion indicating that cells may be capable of forming niches in an autoregulatory manner. *In vitro*, both exogenously controlled parameters, including physical and chemical factors in the culture environment and autocrine and paracrine secretion of endogenously produced factors, mediate the growth and fate decision of stem cells. These exogenous factors include the type and

age of cell: culture environment such as temperature, pH, osmolality, humidity, and the oxygen tension as well as nutrients and toxins (Peerani et al., 2007). Cell culture media have an important impact on growth and differentiation of stem cells. An ideal culture condition would optimally mimic the natural environment of the particular cell type *in vivo*.

The diverse culture conditions utilised for the *in vitro* expansion and differentiation of stem cells influence the gene expression profiles of stem cells and, hence, probably many of the cell properties (Skottman et al., 2006). Most stem cell lines established to date have been directly or indirectly exposed to xenogeneic products during their derivation, expansion, or differentiation *in vitro*. The exposure of stem cells to xenogeneic products increases the risk of graft rejection and severe immune response in the recipient (Bradley et al., 2002; Selvaggi et al., 1997). Xenogeneic immunogen N-glycoylneuraminic acid (Neu5Gc) for which a preformed antibody exists in humans, has been identified from stem cells cultured with xenogeneic products (Martin et al., 2005; Heiskanen et al., 2007). More recently, Sakamoto and co-workers reported the identification of another predominant immunogen apoB-100 that was acquired by stem cells from xenogeneic products in the culture environment (Sakamoto et al., 2007; Hisamatsu-Sakamoto et al., 2008). Other potential risks to the recipient include viral or bacterial infections, prions, and as yet unidentified zoonoses (Cobo et al., 2005). Therefore, for research purposes, as well as for the clinical application of stem cells, the development of a completely defined, xeno-free, and standardised culture conditions is highly desirable.

2.2.1 Culture of adipose stem cells

In most cases, the capacity of adult stem cells to divide *in vitro* is limited, making it difficult to generate large numbers of stem cells. Methods are being developed to grow large quantities of adult stem cells in cell culture and to manipulate them to generate specific cell types so they can be used to treat injury or disease.

ASCs are a rare exception among adult stem cells as they can be isolated in high numbers from either liposuction aspirates or subcutaneous adipose tissue fragments and can be easily expanded *in vitro* (Zuk et al., 2001; Lindroos et al., 2009). The isolation of ASCs is straightforward utilizing manual mincing and collagenase I enzymatic digestion (Zuk et al., 2001). Currently, the standard *in vitro* expansion of ASCs utilises fetal bovine serum (FBS) and various other xenogeneic reagents such as trypsin, serum albumin, and growth factors (Lindroos et al., 2009). The species of origin and the concentration of the serum affect the proliferation of ASCs (Kocaoemer et al., 2007; Mirabet et al., 2008; Herrera and Inman, 2009). In fact, replacing FBS with pooled allogeneic human serum (alloHS) and human serum derivatives leads to equal or higher proliferation rates and multilineage differentiation capacity of ASCs (Katz and Parker, 2006; Kocaoemer et al., 2007; Mirabet et al., 2008). When cultured in the presence of serum, ASCs do not require any feeder cells or extracellular matrix (ECM) to aid in the attachment. Serum composition is largely uncharacterised, containing variable amount of cytokines and growth factors, and showing significant lot to lot variability, which may affect the reproducibility of the results (Caterson et al., 2002; Herrera and Inman, 2009; Lindroos et al., 2009). Autologous human serum (autoHS) is a feasible option for

clinical applications, because it eliminates the problem of introducing xenogeneic or allogeneic antibodies into the recipient (Mesimäki et al., 2009). The use of autologous human serum for clinical applications is hindered, however, due to the limited availability of the large quantities needed for *in vitro* expansion of the cells.

The drawbacks of the use of serum and the risks related to the use of xenogeneic products have lead to the development of serum-free media formulations. As a result, reduced serum media (Parker et al., 2007), serum-free media (Koller et al., 1998; Meuleman et al., 2006; Qizhou et al., 2007) and xeno-free media (Lindroos et al., 2009) are now available for adult stem cell expansion.

2.2.2 Culture of human embryonic stem cells

Human ESCs tend to maintain tight contacts with their neighbors and grow in colonies in culture. Human ESCs are difficult to maintain *in vitro* because they tend to follow their natural cell fate and differentiate spontaneously. Most culture conditions result in some level of unwanted spontaneous differentiation of hESCs. Differentiation is a result of many complex interactions with intrinsic and extrinsic factors, including growth factors, ECM molecules and components, environmental stressors, and direct cell-to-cell interactions (Peerani et al., 2007). While some spontaneously differentiated cells usually appear at the margin and at the centre of hESCs colonies, an ideal culture condition provides growth support with minimal amounts of differentiated cells. Since the first establishment of permanent hESC line in 1998 (Thomson et al., 1998), various culture conditions have been described for the derivation and expansion of hESCs. Some of these culture methods are presented in Tables 1 and 2.

Feeder cell-dependent culture conditions

Initially, the establishment of hESC lines utilised mitotically inactivated MEFs as feeder cells and FBS-containing culture medium for both feeder cells and hESCs (Thomson et al., 1998). MEFs have a limited lifespan in culture and can only be cultured for five to six passages before entering senescence (Choo et al., 2006). To eliminate xeno-contamination, human feeder cells have replaced mouse feeder cells to support the undifferentiated growth of hESCs (Table 1). Compared to MEFs, human feeder cells have an extended lifespan (Amit et al., 2003). Human ESC lines can maintain their self-renewal and pluripotency on several types of human feeder cells including hFFs (Amit et al., 2003; Hovatta et al., 2003), fetal placental fibroblasts (Genbacev et al., 2005), uterine endometrium cells (Lee et al., 2005), adult marrow stroma cells (Cheng et al., 2003), fetal or adult muscle and skin cells (Richards et al., 2002; Richards et al., 2003), and autologous hESC-derived fibroblast cells (hESC-df; Xu et al., 2004; Wang et al., 2005; Stojkovic et al., 2005). Recently, basic fibroblast growth factor (bFGF)-secreting hESC-df cells were derived enabling hESC cultures to be maintained without exogenous bFGF (Saxena et al., 2008; Unger et al., 2009). Although hESC-df cells provide an interesting opportunity, differentiation of fibroblast-like cells from hESCs and their use for hESC maintenance is very labor intensive and not an optimal choice for standardisation and mass production of undifferentiated hESCs.

Different types of human feeder cells appear to have different capabilities to support the growth of undifferentiated hESCs (Richards et al., 2003; Eiselleova et al., 2008), and MEFs seem to support the growth of some hESC lines better than human feeder cells (Richards et al., 2002; Richards et al., 2003). The mechanisms by which feeder cells form a supportive niche for the maintenance of undifferentiated hESCs in culture are not entirely understood, but feeder cells are suggested to provide a suitable attachment substrate for hESCs and to secrete important soluble factors (Raikwar et al., 2006). To optimise the culture conditions, great effort has been made to identify conditioned media (CM) components for hESC self-renewal. High throughput screening methods have been used to investigate the protein composition of media conditioned by mouse and human feeder cells (Lim and Bodnar, 2002; Prowse et al., 2005; Prowse et al., 2007), providing preliminary insight into the possible feeder cell-secreted factors that support hESC growth. Human feeder cells secrete transforming growth factor beta 1 (TGFβ1), Activin A, bFGF, and low levels of bone morphogenic protein 4 (BMP4), while mouse feeder cells secrete comparable levels of TGFβ1 and BMP4, higher levels of Activin A, and no bFGF (Lim and Bodnar, 2002; Prowse et al., 2005; Prowse et al., 2007; Eiselleova et al., 2008). Most feeder cells have been exposed to xenogeneic products such as FBS, during their isolation and culture. However, the establishment of a xeno-free hFF cell line using human serum has been reported (Ellerström et al., 2006; Meng et al., 2008). Although, several xeno-free medium formulations have been developed for the derivation and propagation of primary cell lines, such as fibroblasts, the performance of these media for the expansion of fibroblasts is poor (K.R. unpublished observations).

Initially, FBS was used in the culture medium for hESCs. FBS, however, had a negative effect on hESCs as the colonies undergo excessive differentiation (Amit et al., 2000; Amit and Itskovitz-Eldor, 2002). The development of a commercially available serum replacement, Knockout™ serum replacement (KO-SR, Invitrogen) was a major advancement in the establishment of a serum alternative (Price et al., 1998). KO-SR supplemented with bFGF supports the prolonged growth of hESCs in an undifferentiated state, with a higher growth rate and cloning efficiency than in FBS-containing medium (Amit et al., 2003; Richards et al., 2003; Koivisto et al., 2004). To further examine the mechanisms that support the enhanced growth of hESCs in KO-SR-containing medium, the gene expression profiles of hESCs cultured under FBS- and KO-SR-containing media formulations were examined (Skottman et al., 2006). Although the expression of stem cell markers and their differentiation capacity in EBs were similar in both conditions, surprisingly, over 100 genes were significantly differentially expressed in these conditions. Further, many differentially expressed genes in cells cultured in medium containing serum included those expressed in differentiated cells. Such changes may have fundamental importance for hESCs and as many of the differentially expressed genes have no known biological function, further studies are required to clarify the true impact of these results. KO-SR supplemented with bFGF also supports the derivation of hESC lines (Genbacev et al., 2005; Inzunza et al., 2005) and currently KO-SR is widely used for the derivation and culture of hESCs. Although the use of KO-SR in hESC culture medium provides more standardised and more defined culture conditions compared to FBS-containing conditions, it contains AlbuMAX, a lipid-rich bovine serum albumin (BSA) and bovine transferrin and is a xenogeneic component (Price et al., 1998).

Table 1. Human feeder cell- dependent culture methods for hESCs.

Feeder-cell source	Medium components	M/D	References
Fetal muscle, Fetal skin, Adult fallopian tube	FBS/Human serum	M/D	Richards et al. 2002
Adult skin, Adult muscle	FBS/KO-SR, bFGF	M	Richards et al. 2003
Human foreskin	FBS, LIF	M/D	Hovatta et al. 2003
Human foreskin	KO-SR, bFGF	M	Amit et al. 2003
Adult bone marrow stroma	KO-SR, bFGF	M	Cheng et al. 2003
hESC-derived fibroblasts	KO-SR, bFGF	M	Xu et al. 2004
Human foreskin	KO-SR, bFGF	M/D	Inzunza et al. 2005
Placenta	KO-SR, bFGF	M/D	Genbacev et al. 2005
Uterine endometrium	KO-SR, bFGF	M/D	Lee et al. 2005
hESC-derived fibroblasts	KO-SR, bFGF	M/D	Wang et al. 2005
Xeno-free human foreskin	Human serum	M/D	Ellerström et al. 2006
Human foreskin	HEScGRO	M	Chin et al. 2009
Human foreskin	KO-SR XF	M	Chin et al. 2009

Abbreviations: M=Maintenance; D=Derivation; HEScGRO=Chemically defined xeno-free medium (Millipore); KO-SR XF= Chemically defined xeno-free medium (Invitrogen). Other abbreviations are presented beginning at page 13.

Human serum has also been used as a xeno-free alternative for the maintenance and derivation of hESCs (Richards et al., 2002). In 2006, Ellerström and co-workers reported the establishment of xeno-free hFF feeders for hESC derivation in a medium supplemented with human serum and devoid of any animal-derived material (Ellerström et al., 2006). In accordance with previously published data (Richards et al., 2002), Ellerström et al. (2006) also reported the ability to derive hESCs in human serum-containing medium using a xeno-free derivation procedure with continuous propagation of undifferentiated cells for more than 30 passages. Although human serum provides a xeno-free serum alternative, this source, similar to FBS, is plagued by batch variability, a poorly defined composition, and variable efficacy in hESC cultures (Richards et al., 2002).

Feeder cell-free culture conditions

Significant progress has been made in the development of feeder cell-free culture methods for hESC propagation (Table 2). The first feeder cell-free maintenance method for existing hESC lines were cultures on Matrigel using CM from MEFs (Xu et al., 2001). Matrigel is a complex basement membrane mixture secreted by mouse sarcoma cells, composed of several ECM components such as laminin, collagen IV, entactin, and heparan sulfate proteoglycan, as well as various growth factors. There are previous reports of the use of FBS coating (Vallier et al., 2005; Soh et al., 2007) and human serum coating (Stojkovic et al., 2005) as a matrix for feeder cell-free propagation of hESCs. Although the media used with the FBS coating was defined in both reported studies, the serum used as a coating material was not, and therefore more defined matrix materials for the feeder cell-free propagation of hESCs are needed. As a step forward, human derived ECM components such as laminin or fibronectin have also been used as substrates in feeder cell-free culture conditions (Xu et al., 2001; Amit et al., 2004; Li et al., 2005; Beattie et al., 2005; Noaksson et al., 2005; Liu et al, 2006; Lu et al., 2006). They have, however, proven to be inferior compared to Matrigel in the long-term hESC

cultures, thus indicating that other yet unknown factors are needed for successful feeder-cell free culture of hESCs. Recently, ECM derived from xeno-free hFFs was described to support the maintenance of hESCs for at least 20 passages (Meng et al., 2008).

Table 2. Feeder cell-free culture methods for hESCs.

Substrate	hESC medium composition	M/D	References
Matrigel/Laminin	CM (MEFs), KO-SR, bFGF	M	Xu et al. 2001
Matrigel	CM (hESC-dF), KO-SR, bFGF	M	Xu et al. 2004
Matrigel	KO-SR, bFGF, BIO	M	Sato et al. 2004
Fibronectin	KO-SR, TGFβ1, bFGF	M	Amit et al. 2004
Matrigel	KO-SR, Noggin, high bFGF	M	Xu et al. 2005
Matrigel/Laminin	X-Vivo 10, high bFGF, SCF, FL, LIF	M	Li et al. 2005
Laminin	X-Vivo 10, high bFGF	M	Genbacev et al. 2005
Laminin	KO-SR, bFGF, Activin A, KGF, NIC	M	Beattie et al. 2005
Fibronectin	CM (MEFs), KO-SR, bFGF	M	Noaksson et al. 2005
Matrigel	PDGF, S1P	M	Pebay et al. 2005
ECM from MEFs	KO-SR, Plasmanate, LIF, bFGF	M/D	Klimanskaya et al. 2005
Human serum matrix	CM (hESC-df), KO-SR, bFGF	M	Stojkovic et al. 2005
FBS matrix	CDM (Activin A/Nodal, bFGF)	M	Vallier et al. 2005
Matrigel	KO-SR, Noggin, high bFGF	M	Wang et al. 2005
Matrigel/Fibronectin	N2, B27, high bFGF	M	Liu et al. 2006
Matrigel	N2, B27, BSA, bFGF	M	Yao et al. 2006
Matrigel/Fibronectin/ Fibronectin, Collagen	HESCO (bFGF, Wnt3a, April/BAFF)	M	Lu et al. 2006
Matrigel	KO-SR, bFGF, BDNF, NT3, NT4	M	Pyle et al. 2006
Matrigel/ Human ECM mix	mTeSR1/TeSR1	M/D	Ludwig et al. 2006
Laminin	SFM, high bFGF	M/D	Fletcher et al. 2006
Matrigel/FBS matrix	CDM (Activin A, bFGF, PTN)	M	Soh et al. 2007
Matrigel	X-Vivo 10, high bFGF, TGFβ1	M	Peerani et al. 2007
Matrigel	KO-SR, IGF-II	M	Bendall et al. 2007
Matrigel	DC-HAIF (HRG-β1, Activin A, IGF-I, bFGF)	M	Wang et al. 2007
None	NC-CM and hEL-CM	M	Bigdeli et al. 2007
ECM from XF-hFFs	HEScGRO, high bFGF, ROCK	M	Meng et al. 2008
Fibronectin	StemPro	M	Chin et al. 2009
Matrigel	Stemedia™ NutriStem™ XF/FF	M	Stemgent

Abbreviations: M=Maintenance; D=Derivation; X-Vivo 10=Xeno-free basal medium; CDM=chemically defined medium; HESCO=chemically defined medium; Human ECM mix=laminin, fibronectin, vitronectin, collagen; mTeSR1=chemically defined medium, containing xeno-derived components; TeSR1=chemically defined xeno-free medium; SFM=Serum-free medium; DC-HAIf=chemically defined medium; HEScGRO=Chemically defined xeno-free medium (Millipore); Stempro=defined, serum-free medium (Invitrogen); Stemedia™ NutriStem™ XF/FF= defined, serum-free medium (Stemgent). Other abbreviations are presented beginning at page 13.

KO-SR is also widely used as a culture medium in feeder cell-free methods (Table 2). The early feeder cell-free studies relied on the use of CM from MEFs containing KO-SR (Xu et al., 2001; Noaksson et al., 2005). Later, feeder cell-free propagation of hESCs using CM from hESC-derived fibroblasts was described (Xu et al., 2004; Stojkovic et al., 2005). CM is usually prepared by overnight incubation with a confluent feeder layer and by subsequent filtration prior to its use (Bigdeli et al., 2007). CM from different feeder cells all differ in their ability to support the growth

of undifferentiated hESCs (Xu et al., 2001). Feeder cell-free and matrix-free cultures of hESCs directly on plastic surfaces have also been described (Bigdeli et al., 2007). These studies used extensive adaptation processes in which hESCs cultured on Matrigel were first adapted to neonatal chondrocytes-CM (NC-CM) during a 20-day cultivation period and subsequently transferred to cell- and matrix-free polystyrene dishes where hESCs were cultured for 11 days in the presence of NC-CM followed by culture in the presence of human embryonic lung fibroblast CM (hEL-CM) for 43 passages. For some unknown reason, the hESCs were dependent on the use of NC-CM during the adaptation process.

Feeder cell-free derivation of hESC lines is also possible. ECM from MEFs and KO-SR containing medium were used in the first successful feeder cell-free derivation (Klimanskaya et al., 2005). Another report described the derivation of two hESC lines in a defined culture medium, TeSR1, containing components solely derived from recombinant sources or purified from human material, on substrate formed by human ECM components: fibronectin, laminin, collagen IV, and vitronectin (Ludwig et al., 2006). One of these lines had karyotype 47XXY, however, which is common in human embryos. The second line gained an extra chromosome 12 at passage level 40. Currently, the modified formulation, mTeSR1, containing xenogeneic components, is one of the most commonly used feeder cell-free culture systems utilised with Matrigel. Recently, Fletcher and co-workers compared different derivation conditions for hESCs (Fletcher et al., 2006). They used human laminin, Matrigel, or hDFs as the substrate and KO-SR containing medium or serum and xeno-free medium (SFM), which were either conditioned with hDFs or used without conditioning. They were able to derive one hESC line using nonconditioned SFM and human laminin in xeno-free derivation conditions. In response to embryonic cell lifting, the line was transferred on hDFs only 8 days after initial derivation on human laminin. For unknown reasons, expression of the undifferentiated hESC marker SSEA-4 was gradually lost in this line maintained in SFM.

Chemically defined and xeno-free culture conditions

To develop a chemically-defined culture environment that maintains undifferentiated self-renewal of hESCs, it is necessary to first determine the signals and mechanisms governing hESC fate. Over the last few years, a number of molecular factors and signalling pathways that play a major role in maintaining hESCs self-renewal were identified (Figure 2). One of these important factors is bFGF, which seems to act in part by inducing the expression of TGF-β superfamily molecules and insulin-like growth factor (IGF)-II (Xu et al., 2005; Bendall et al., 2007), activation of Gremlin-1, and suppression of BMB4 (Greber et al., 2007). Basic FGF is the most commonly used media component required for hESC maintenance, both in the presence and absence of fibroblast feeder cells. TGFβ superfamily members, namely TGFβ, Activin A, and Nodal, are also important regulators of hESC self-renewal by inducing phosphorylation of the intracellular mediators Smad2 and/or Smad3 and have been used by several research groups in the feeder cell-free culture of hESCs (Amit et al., 2004; Beattie et al., 2005; James et al., 2005; Vallier et al., 2005; Peerani et al., 2007). Furthermore, Activin A induces the expression of several pluripotency genes, such as Oct-4 and Nanog, and stimulatory growth factors, such as Nodal, Wnt3, bFGF, and FGF8 (Xiao et al.,

2006). More complex is the role of the Wnt family of proteins in hESC self-renewal because of conflicting reports regarding its role in hESCs self-renewal (Dravid et al., 2005). A proposed Wnt-signalling activator, glycogen synthase kinase-2 inhibitor, BIO, as well as a combination of Wnt3a, April/BAFF and bFGF have been used to supplement feeder cell-free culture of hESCs (Sato et al., 2004; Lu et al., 2006). Inhibiting the activity of BMPs with a specific BMP signalling antagonist such as Noggin may also enhance the maintenance of hESCs in a pluripotent state (Wang et al., 2005; Xu et al., 2005). IGF-II is implicated as a direct mediator of hESC growth, being expressed in response to bFGF, and in parallel with TGFβ/Nodal/Activin (Bendall et al., 2007). bFGF might drive the expression of IGF-II and TGFβ factors from the hESC-df-like cells generally surrounding the hESC colonies in both feeder and feeder cell-free cultures. Another study described the importance of IGF-I for hESCs self-renewal, as well as a role for heregulin-β1 (HRG-β1) an human EGF receptor 2/3 ligand (ErbB), and Activin A using a simple defined medium (Wang et al., 2007). Neurotrophins (NT; Pyle et al., 2006), pleiotrophin (PTN; Soh et al., 2007), and a Rho-associated kinase inhibitor (ROCK) (Watanabe et al., 2007) may be used to enhance clonal growth of hESCs by inhibiting apoptosis.

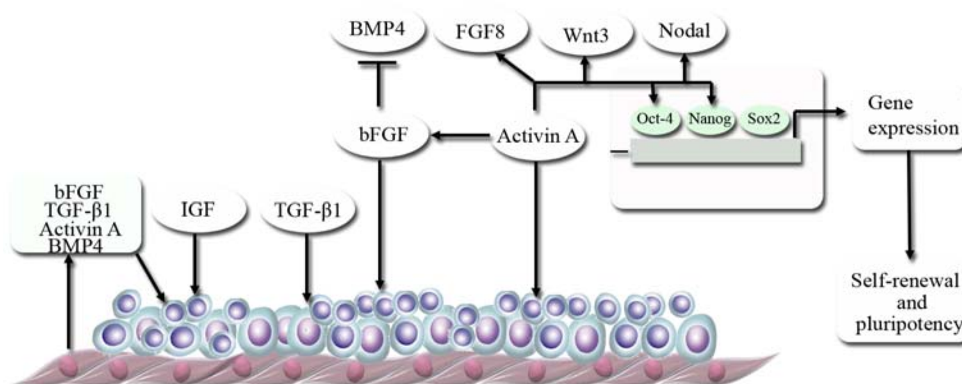

Figure 2. Molecules regulating pluripotent stem cell renewal. Growth factors such as bFGF, IGF, TGF-β1, and Activin A stimulate pluripotent stem cell growth via direct and indirect mechanisms, whereas BMP signalling induces premature differentiation.

Besides growth factor proteins, recent reports indicate that lipid molecules, abundant in serum and AlbuMAX, which is an essential ingredient within KO-SR (Price et al., 1998), are also important regulators for maintaining hESC self-renewal. Sphingosine-1-phosphate (S1P) enhances proliferation and prevents apoptosis of hESCs (Inniss and Moore, 2006), whereas S1P and platelet-derived growth factor (PDGF) synergistically stimulate the self-renewal of hESCs grown on MEFs (Pebay et al., 2005; Wong et al., 2007). Albumin-associated lipids present in KO-SR have a strong effect on hESC self-renewal (Garcia-Gonzalo and Izpisua Belmonte, 2008). In this study, the major lipids bound to the lipid-rich albumin were identified and several lipid candidates: lysophosphatidylcholine, lysophosphatidic acid (LPA), S1P, prostaglandin (PG) E_2, and dexamethasone were tested in the culture of hESCs, but the active lipid responsible for the positive effect of AlbuMAX on hESC self-renewal remains to be discovered. Previously LPA has been shown to inhibit neuronal differentiation of hESCs by activating the Rho/ROCK and the PI3/Akt pathways (Dottori et al., 2008).

Human ESCs can be cultured under feeder cell-free conditions, thus it is possible to systematically define and characterise both the required exogenous factors and the factors produced by supportive feeder cell layers. Several chemically defined culture media with a cocktail of recombinant growth factors and cytokines have been developed to sustain undifferentiated propagation of existing hESC lines (Table 2). Feeder-cell free culture conditions are attractive with regard to the therapeutic use of hESCs and for drug testing because of the absence of other cell types that may hamper the utilization of the cells. Feeder cell-free culture of hESCs routinely utilises mostly xenogeneic enzymatic cell dissociation, which is considerably faster and simpler than mechanical dissection and enables large-scale expansion of hESCs. Several reports however indicate that the use of enzymes may increase the risk of introducing cytogenetic aberrations during propagation *in vitro* due to excessive and/or frequent dissociation to single cells (Draper et al., 2004; Mitalipova et al., 2005; Imreh et al., 2006). In contrast, mechanical dissociation allows for selective transfer of exclusively undifferentiated cell aggregates and seems to better maintain genetic stability (Lei et al., 2007).

Currently, there are at least four commercially available xeno-free culture media, HEScGRO (Millipore), developed for the culture of hESC lines with feeder cells; KnockOut™ SR Xeno-free (KO-SR XF, Invitrogen) for feeder or feeder cell-free culture of hESCs; Stemedia™ NutriStem™ XF/FF (Stemgent); and TeSR2 (StemCell Technologies) for feeder-cell free culture of hESCs. Whereas TeSR2 and Stemedia™ NutriStem™ XF/FF media are designed to be used with Matrigel, KO-SR XF can be used with a humanised matrix, CELLstart, thus enabling xeno-free feeder cell-free culture of hESCs.

2.2.3 Culture of induced pluripotent stem cells

Human iPS cells exhibit many features characteristic of hESCs including morphology, marker expression with minor differences, and differentiation potential; hence, human iPS cells have similar culture requirements as hESCs. Similar to hESCs, however, xenogeneic products are present at multiple steps in the currently available protocols for iPS cell generation and maintenance. First, the primary cultures of human somatic cells to be reprogrammed are usually established using FBS-containing media and passaged with xenogeneic enzymes such as trypsin. In addition, cell lines used to produce viral supernatants for the reprogramming of somatic cells are usually maintained with FBS and trypsin. Finally, in the culture of iPS cells, MEFs or human feeder cells are routinely used with FBS or KO-SR containing culture medium and cells are dissociated using enzymes from animal origin (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008; Lowry et al., 2008). Feeder cell-free culture of human iPS cells on Matrigel using CM derived from MEFs has also been reported (Aasen et al., 2008).

So far, the derivation of iPS cell lines has relied on the use of MEF feeder cells but recently human iPS cells were derived on immortalised human fibroblast feeders from their parental line using KO-SR containing medium in the derivation and subsequent culture (Unger et al., 2009). Another recent report describes the feeder cell-free derivation of human iPS cell lines on Matrigel using MEF-CM or serum and feeder layer-free culture medium (Totonchi et al., 2009). More recently, the

generation of iPS cells under xeno-free conditions has been reported; xeno-free hDFs were derived using human serum and were used as both the source of cells for reprogramming as well as autologous feeder cells for the generation and maintenance of iPS cells (Rodriguez-Piza et al., 2010). Two different xeno-free culture media containing either KO-SR XF or human plasma-derived cell culture additive were used for the reprogramming of human fibroblasts to pluripotency and subsequent culture of the resulting iPS cells (Rodriguez-Piza et al., 2010). The recent advancements in the generation and maintenance of iPS cells should facilitate the safe clinical translation of iPS cell-based technology.

2.2.4 Culture of stem cells under hypoxia

The growth, differentiation, and fate decisions of stem cells and multipotent progenitors are influenced by complex signals in the microenvironment, including oxygen availability. Oxygen is a potent signalling molecule that has received increasing recognition for its ability to affect fundamental characteristics, such as self-renewal and differentiation, of various stem cell types (Covello and Simon, 2004; Covello et al., 2006).

The response to hypoxia is regulated by hypoxia inducible factors (HIFs), which regulate the expression of over 200 genes, including genes involved in erythropoiesis, apoptosis, and proliferation. HIFs are transcription factors consisting of three oxygen-dependent alpha subunits, HIF-1α, HIF-2α, and HIF-3α, and the constitutively expressed beta subunit HIF-1β. Hypoxia-inducible factors exhibit reduced stability as the oxygen concentration rises and are rapidly degraded under atmospheric oxygen tensions (Semenza, 2000). They regulate transcriptional responses as heterodimers with HIF-1β (Wenger and Gassmann, 1997) through binding to cis-acting hypoxia-response elements (HREs) on their target genes when oxygen tension is low (Wenger and Gassmann, 1997; Greijer et al., 2005). HIF-3α differs from HIF-1α and HIF-2α in that it lacks the C-terminal activation domain (CTAD) required for co-activator binding and is thus unable to recruit co-transcriptional regulators and basal transcriptional machinery to gene targets. HIF-2α targets the HRE-sequence independently of HIF-1α, suggesting that they have functionally diverse roles. Indeed, HIF-1α, the first HIF-α subunit described and originally thought to be the global regulator of the hypoxic response (Semenza and Wang, 1992), predominantly regulates glycolytic genes (Hu et al., 2003), whereas HIF-2α regulates hypoxia-induced erythropoietin (Warnecke et al., 2004; Rankin et al., 2007). To date, little is known about which genes are targeted by HIF-3α. The contribution of HIF-2α and HIF-3α to the regulation of the hypoxic response, however, remains to be fully characterised (Forristal et al., 2009).

Since the first derivation of hESCs was reported (Thomson et al., 1998) it has become routine practice to culture these cells under atmospheric oxygen (20% O_2). Naturally, human embryos grow *in vivo* in lower oxygen tension environments and physiological O_2 levels improve *in vitro* embryo development (Thompson et al., 1990; Giles and Foote, 1997; Petersen et al., 2005) and increase cell number, particularly of the ICM (Harvey et al., 2004; Rinaudo et al., 2006) indicating that O_2-regulated gene expression supports the maintenance of pluripotent cells. Thus,

hESCs, a derivative of embryos, will likely grow more favorably in a reduced O_2 tension (Westfall et al., 2008).

Hypoxic exposure promotes the proliferation of mouse ESCs through cooperation of arachidonic acid and PI3K/Akt signalling pathways and increases differentiation via suppression of the leukemia inhibitor factor (LIF)- signal transducers and activator of transcription (STAT)3 signalling pathway (Jeong et al., 2007; Lee et al., 2007). In contrast, there is emerging evidence to suggest that reducing the oxygen concentration towards physiological levels is beneficial for the *in vitro* maintenance of hESCs in terms of decreasing the amount of spontaneous differentiation, supporting self-renewal (Ezashi et al., 2005; Ludwig et al., 2006; Peura et al., 2007; Westfall et al., 2008), and reducing spontaneous chromosomal aberrations (Forsyth et al., 2008). The controversy continues, however, as a recent report suggests that there are no significant advantages of culturing hESCs under reduced oxygen tension (Chen et al., 2009).

The Notch signalling pathway was suggested to be involved in the maintenance of self-renewal under hypoxia (Prasad et al., 2009), but although the Notch signalling pathway regulates the fate of many types of stem cells, there is currently very little evidence for its involvement in controlling the fate of hESCs (Walsh and Andrews, 2003; Babaie et al., 2007). Oct-4, one of the core transcription factors known to be essential for self-renewal, is an HIF-2α-specific target gene (Covello et al., 2006). Recently, microarray analysis performed on hESC lines cultured under hypoxia and normoxia revealed 149 differentially expressed genes (Westfall et al., 2008). Although genes associated with pluripotency, including Oct-4, Sox2 and Nanog were mainly unaffected, some genes controlled by these transcription factors, including left-right determination factor 1 (LEFTY), showed increased expression under hypoxia (Westfall et al., 2008). Another report described the increased expression of Sox2, Nanog, and Oct-4 mRNA as well as Oct-4 protein, and increased proliferation of hESCs under hypoxic conditions (Forristal et al., 2009). Furthermore, they showed that HIF-1α protein was not expressed at a 20% oxygen level and displayed only a transient nuclear localization at a 5% oxygen level, whereas HIF-2α and HIF-3α displayed nuclear localization following long-term culture at a 5% oxygen level and were significantly upregulated compared to cells cultured at a 20% oxygen level. Their results also suggested that while HIF-3α regulates the expression of both HIF-1α and HIF-2α, it is HIF-2α that regulates the pluripotency of hESCs and their proliferation under hypoxic conditions. More recently, it was shown that hypoxic conditions improve the efficiency to generate iPS cells from mouse and human somatic cells (Yoshida et al., 2009). A comprehensive definition of the molecular mechanisms favoring pluripotency under hypoxia, however, has yet to be determined.

2.3 Clinical grade stem cells

2.3.1 Stem cells for regenerative medicine

The basic and clinical research accomplished during the last few years on stem cells constitutes a revolution in regenerative medicine by indicating the possibility of generating multiple therapeutically useful cell types. Cell therapies based on adult stem cells for several disorders have been in use for many decades, beginning in 1968 with the first successful bone marrow transplant (Gatti et al., 1968; Bach et al., 1986; Carpenter et al., 2009). Although few cell-based products, such as Carticel, an autologous chondrocyte, and products for skin replacement therapies, have received the United States Food and Drug Administration (FDA) approval, so far no stem cell products have been approved in the EU or US. There are many ongoing clinical trials involving the direct application of stem cells (see http://clinicaltrials.gov, https://eudract.emea.europa.eu/). Several stem cell products, including human adult MSCs for the treatment of graft versus host disease are currently in late-stage clinical trials (Carpenter et al., 2009) and human autologous ASCs for repairing perianal fistulas are in phase III clinical trials (see http://clinicaltrials.gov). In January 2009, the Geron Corporation announced that the FDA had granted approval to begin clinical trials of its product GRNOPC1, which contains oligodendrocyte progenitor cells derived from hESCs (Geron, 2009a; von Tigerstrom, 2009). This clinical trial will involve the administration of GRNOPC1 to patients with recent, functionally complete spinal cord injuries. As a Phase I trial, its primary objective is to assess the safety of the product, though secondary endpoints will also be used to assess efficacy (Geron, 2009a; von Tigerstrom, 2009). The approval of this investigational new drug (IND) application is a significant milestone in the clinical translation of stem cell research, because it will be the first approved clinical trial of an hESC-derived product. The Geron's IND application, however, was put on clinical hold in May 2009, a few months after it was filed (Geron, 2009b; von Tigerstrom, 2009). In 2008, the International Society for Stem Cell Research (ISSCR) released Guidelines for the Clinical Translation of Stem Cells (ISSCR, 2008; von Tigerstrom, 2009). These guidelines aim to promote an international consensus on important issues surrounding the clinical applications of novel stem cell therapies in response to growing concerns relating to "stem cell tourism" where unproven stem cell treatments not subjected to adequate testing or review are offered in clinics around the world (von Tigerstrom, 2009).

At present, it is unclear which stem cell type will provide the best approach for cell therapy. Depending on the clinical scenario, one cell may be more feasible than another. Even though tissue progenitor cells have limited growth and differentiation potential, they have a lower tendency to form tumors and to produce mixed phenotypes and are thus ideal candidates for tissue engineering of organs. On the other hand, pluripotent stem cells have the ability to grow indefinitely and differentiate into cells of all three germ layers. Ethical concerns regarding the use of embryos, the formation of teratomas, and the potential for evoking an immune response currently dampen enthusiasm for their clinical potential. The ability to reprogram adult somatic cells into iPS cells is a scientific breakthrough, but the use of oncogenes, retroviral transduction, and limited understanding hinders their

clinical potential. The stem cell field is full of challenges, technical, regulatory, and clinical, and there are inherent risks in all cell therapies, whereby the safety of the product will have to be balanced against the life-threatening nature of the indication and the risk of not having an alternative therapy available (Hipp and Atala 2008).

2.3.2 Regulatory requirements for cell therapy products

The use of cells in therapeutic applications requires adherence to the regulatory requirements outlined in Europe in several European Commission directives and European Medicinal Agency's (EMEA) guidelines, whereas in the US cell therapies are regulated under the FDA's Good Tissue Practices Final Rule (US 21 C.F.R., Part 1271; US FDA, 1998). The EU has adopted an advanced therapy medicinal products (ATMPs) regulation that specifically applies to cell therapy products (EC Regulation 1394; EC Directive 2001/83/EC; EC Regulation 726/2004). The EU Directive (2004/23/EC) establishes standards of quality and safety for the donation, procurement, testing, processing, storage, distribution, and preservation of human tissues and cells. The ATMP regulations define tailor-made technical requirements and establish new standards for clinical trials addressing development, manufacture, and quality control, as well as non-clinical and clinical development of cell-based medicinal products. The regulations in both the EU and US divide procedures according to the degree of manipulation involved and the level of risk associated with the cell product. Therefore, the interpretation of the regulation is to some extend based on the nature of the cell product being manufactured. In the EU, minimal manipulation products, such as cryopreservation of cells, will only be subjected to limited requirements and may be produced according to defined industrial standards, known as good tissue practices (GTPs). More than minimally manipulated cell products require elevated control and adherence to current good manufacturing practices (cGMPs; EC Directive 2001/83/EC; 2004/23/EC; US 21 C.F.R., Part 1271). The presumption is that for minimally manipulated cell products, the primary concern is with contamination and communicable disease, whereas cells that undergo more extensive manipulation will raise a broader range of safety issues and questions about whether they will perform the intended function in the human recipient (von Tigerstrom, 2009).

GMP is a quality assurance system used in the pharmaceutical industry covering all stages of the manufacture from the isolation of cells to storage of the cell product. The objectives of GMPs are to identify and standardise the best practices that result in safe products with reproducible characteristics, quality, and overall safety, as well as protecting the wellbeing of the patient. The quality management framework defined under GMP should ensure quality and traceability of raw materials, personnel qualifications with defined responsibilities, validation of equipment, facility, processes and quality control methods, standard operating procedures for all functions, record keeping, error management, standard production and process controls to monitor both quality and safety, as well as constant review of the quality of the activities by self-auditing (Unger et al., 2008; Carpenter et al., 2009).

The goal of manufacturing a stem cell-based product is the consistent and reproducible generation of sufficient numbers of cells in an aseptic environment with adequate safeguards and traceability. Consistency of the manufacturing process

can be achieved by minimizing variability at all steps of the manufacturing process and is related to the use of standardised reagents and source material, reproducible expansion and differentiation protocols, and validated in-process and quality control tests. Defining the consistency of the cell-based product is inherently challenging because, unlike molecules, cells are living entities and change over time. Demonstrating of the quality and safety of the stem cell-derived products requires evaluation of the starting materials, demonstration of reproducible expansion and differentiation in an aseptic environment, and assessment of the product characteristics using validated testing methods. Testing the end product is not sufficient as the quality must be built into the product by performing testing throughout the manufacturing process. Phenotypic features such as identity, viability and doubling time, genotypic features such as genomic and epigenetic stability, as well as functional assessment including differentiation potential and safety tests such as purity, sterility, endotoxins and mycoplasma contamination should be tested at critical control points during the manufacturing process. The purity of the cell-based product consists of the active cell type and contaminants such as supporting or accessory cells, undifferentiated cells, or other cells unrelated to the therapeutic activity. The manufacturing process should produce product lots containing consistent amounts of the active cell type and of the contaminants. The relevant tests for the characterization of the final product will depend on the cell population and its mechanism of action, but should include the evaluation of identity; genetic stability and safety; characterization of *in vivo* properties, such as biodistribution, tumorigenicity, toxicity, and immunogenicity; as well as functional evaluation of the cell product, including functional stability. Surrogate measures of efficacy and potency should be developed to allow prediction of the activity of the cell-based product *in vivo* (Carpenter et al., 2009). An overview of the manufacturing process and quality control of clinical-grade stem cell-based products and operations fundamental to cGMP regulations are presented in Figure 3.

Similar to small molecule-based drugs, stem cell products will need to demonstrate potency, efficacy and, most importantly, safety in appropriate animal models before entering clinical trials on humans (Vogel, 2005; Parson, 2006; Hentze et al., 2007). Both in the EU and US, the initiation of clinical trials requires approval by the relevant agency. The application for this approval must be supported by preclinical data showing sufficient preliminary evidence of safety and efficacy, as well as data from any clinical trials already performed (von Tigerstrom, 2009). Many countries have an exemption that may allow unapproved products to be administered to individual patients outside of a clinical trial on a case-by-case basis, but these involve also some oversight by the regulatory agency (EC Directive 2001/83/EC; FDA Regulation, 21 C.R.F., Part 312, § 312.34). Generally, these specific regulations apply when a patient is seriously ill and the need for access to an investigational product can be justified, usually because all conventional therapies have failed (von Tigerstrom, 2009). Strengthening long-term monitoring is also important in the context of stem cell-based products. The European ATMP regulations include specific requirements for post-authorization follow-up and traceability (EC Regulation 1394; EMEA 2008; von Tigerstrom, 2009). The regulatory framework worldwide regarding human cell-derived products is constantly under development and globally there is widespread recognition of the need for an internationally unified regulatory framework to ensure high standards of quality and safety with respect to cell and tissue transplantation.

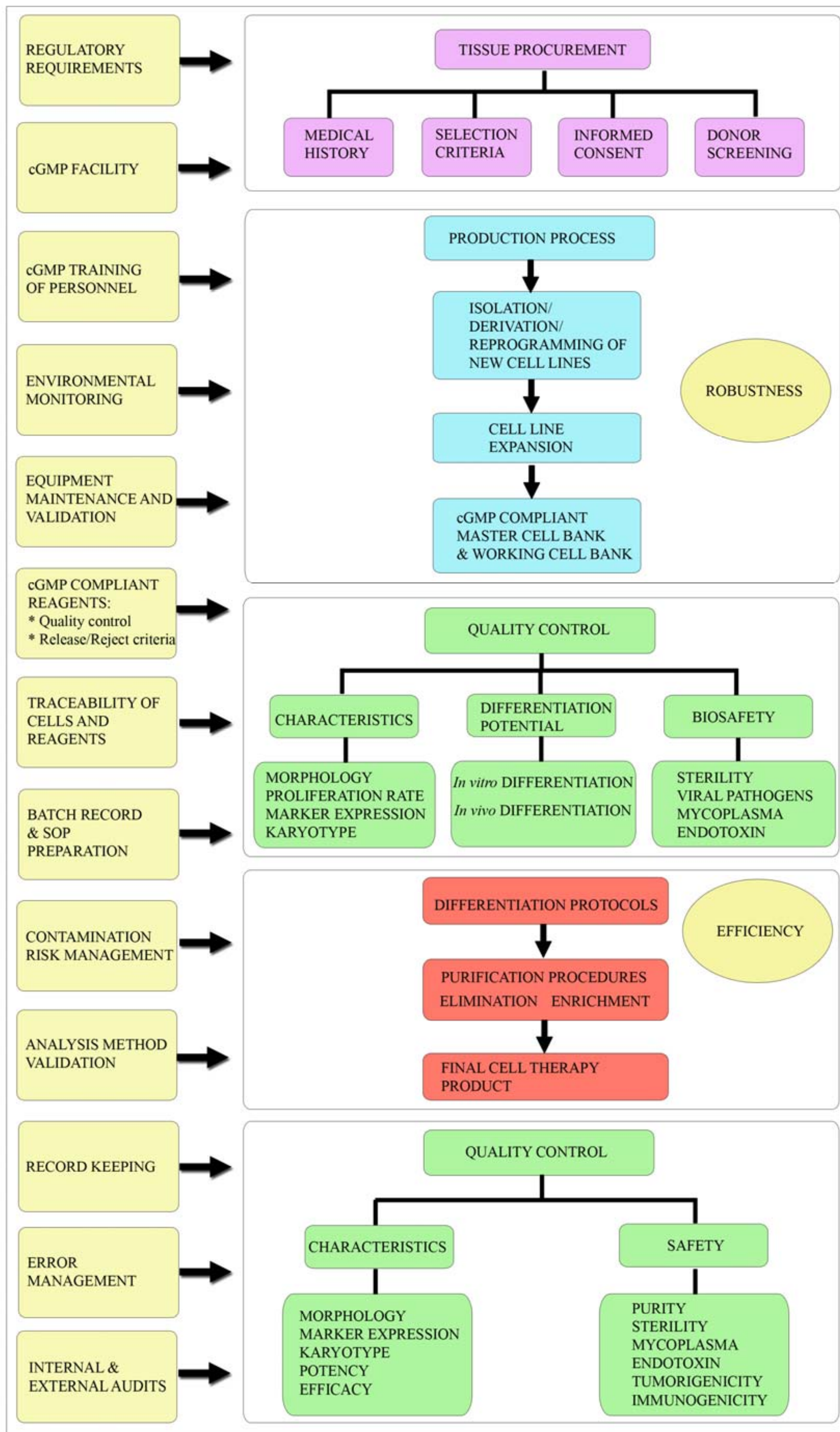

Figure 3. Outline of a general strategy to generate a clinically compliant cell therapy product and operations fundamental to cGMP regulations

2.4 Challenges to the clinical use of stem cells

2.4.1 Culture conditions

The use of defined and xeno-free conditions is the ultimate goal for studies aimed at understanding the regulation of self-renewal and the generation of therapeutically useful cell-based products. Reagents used in the production processes need to be quality assured and traceable. The reagents currently in use for stem cell derivation and propagation, however, have not yet been validated for human or therapeutic use. Therefore, it is essential that we use strict fit-for-purpose justifications, selection criteria, and product specifications to ensure the highest possible product quality and to ensure that suppliers follow an appropriate quality assurance program (Stacey, 2004; De Sousa et al., 2006). Recently, Crook and co-workers published the generation of six clinical-grade hESC lines in ES Cell International Singapore (Crook et al., 2007). These lines were established and banked in a clinical-grade manufacturing process facility in compliance with international regulatory requirements. Interestingly, the lines were derived and propagated using FDA-approved hFFs initially derived and propagated in cGMP-grade FBS-based medium as feeder cells and the hESC lines were derived using cGMP-manufactured KO-SR containing medium. Although qualified xenogeneic reagents have previously received approval for skin replacement cell therapy by the FDA, the overall risk of these topical somatic cell therapy applications is considered extremely low (Hentze et al., 2007), which is not the case with stem cell-based therapies. Recently, the FDA approved Geron's hESC-based product using xenogeneic components for human clinical trials. Because cells can incorporate animal components, however, including the nonhuman sialic acid Neu5Gc and apoB-100, and given the concerns about possible infection and immune responses from xenogeneic products, it is preferable to maintain the cells in defined, xeno-free conditions. Albeit rare, severe anaphylaxis and immune reactions have been reported in patients transplanted with human cells exposed to xenogeneic products (Selvaggi et al., 1997; Mackensen et al., 2000), which may also adversely impact engraftment and therapeutic efficacy (Sakamoto et al., 2007; Hisamatsu-Sakamoto et al., 2008). Therefore, it is likely that when defined and xeno-free culture reagents are available in the future, xenogeneic components will no longer be acceptable for the production of clinical cell-based products from a regulatory point of view. By replacing xenogeneic products with defined xeno-free media, the safety, reproducibility, and quality of the cells with therapeutic potential may be greatly enhanced.

2.4.2 Scale up of production processes

One of the most basic problems is how to generate the very large numbers of cells required for tissue replacement. For clinical application of stem cells, culturing techniques need to be scaled up for robust mass production of clinically relevant quantities of the specified cells. Automated mechanical passaging of hESCs incorporating the advantages of mechanical dissection without sacrificing the practical benefits of enzymatic passaging has been developed (Joannides et al., 2006). The first completely automated robotic system enables plating, feeding and

harvesting hESCs in MEF co-culture (Terstegge et al., 2007). Suspension culture, although difficult to establish with adherence-dependent cells, would offer a robust and scalable expansion of stem cells (Cormier et al., 2006). Proof-of-concept has also been demonstrated using bioreactors for hESCs (Cameron et al., 2006). Further implementation of bioengineering strategies is required to overcome the cell-manufacturing challenges for future clinical cell-based therapies.

2.4.3 Differentiation of stem cells

To realise the full potential of stem cells, it is essential to be able to direct the development of these cells along specific pathways and to control their differentiation. Embryology offers important insights into key pathways regulating pluripotent stem cell differentiation leading to the development of protocols for the efficient generation of a broad spectrum of cell types *in vitro*. The identification of precursor populations for many tissues led to the development of multistep differentiation protocols for the derivation of individual lineages, where the formation of progenitors via a number of intermediate stages is induced by altering culture conditions and by adding specific growth factors and cytokines (Hipp and Atala, 2008; Mountford, 2008; Murry and Keller, 2008). It is well known that different hESC lines and probably iPS cell lines exhibit considerable differences in their differentiation capacity (Osafune et al., 2008). In addition, hESCs and iPS cells tend to differentiate towards some lineages better than others, as neural lineages can be derived with a relative high efficiency (up to 90% of cultures), but a rather low percentage of hESCs, only 1% to 20%, differentiates towards the haematopoietic lineage (Lowell et al., 2006).

The therapeutic potential of stem cells largely relies on efficient and controlled differentiation towards a specific cell type and the generation of homogenous cell populations. One concern about the clinical potential of stem cells is their tendency to form mixed phenotypes in most differentiation protocols (Hipp and Atala, 2008). On the other hand, mixed phenotypes might be a requirement for the efficient differentiation of fully mature phenotypes, through a yet largely unknown complex interaction between the different cell types. Thus, characterising and understanding mixed populations of progenitor stages will be of increasing importance in stem cell research. Genetic manipulation of pluripotent cells may be a way to efficiently achieve unilineage differentiation (Keller, 2005; Hentze et al., 2007). One prerequisite for cell therapy use is the enrichment of the desired cell type from other unwanted lineages and the selective elimination of potentially harmful tumorigenic cells, which will be discussed later (Hentze et al., 2007).

Even if cells can be reliably differentiated into sufficiently homogenous cultures, cells differentiated from pluripotent stem cells tend to resemble fetal rather than adult forms of cells. In many cases, maturation is partial and the differentiated cells share only some of the characteristics of their endogenous counterparts. While lineage-specific gene expression and cell surface markers are commonly used to describe a differentiated phenotype, it is difficult to determine whether differentiated cells are, for example, bona fide neurons or merely neuronal-like cells. The extent to which immature cells will be clinically functional and whether they will mature further *in vivo* remain unknown at this time (Hipp and Atala, 2008; Mountford,

2008; Murry and Keller, 2008). Thus, considerably more work is required to standardise differentiation methods and to reproducibly produce truly functional cell populations for therapeutic use. The next challenge will be to demonstrate the functional utility of these cells, both *in vitro* and in preclinical models of human disease (Murry and Keller, 2008).

2.4.4 Immune rejection

Allogeneic cell transplantation may result in graft rejection and immune reactions and therefore may require lifelong immunosuppression, which can have serious side effects, including an increased tumorigenicity risk (Muruve and Shoskes, 2005). The major advantage of using iPS and adult stem cells for cell therapy is that they offer the potential to generate patient-specific cells that would be recognised as "self" by the immune system, thus preventing rejection of cell transplants. These cells, however, would likely only be produced after the patient becomes ill, precluding their use in the acute phase of the disease. Although cell transplants may not have the full antigenicity of solid organs, there is now clear evidence that hESCs are immunogenic and will be rejected in an allogeneic setting (Grinnemo et al., 2008). Furthermore, once transplanted, hESC derivatives have increased expression of major histocompatibility complex (MHC) antigens, which are further modulated by inflammatory cytokines such as interferon gamma (Murry and Keller, 2008).

Several methods have been proposed to avoid the problem of immune rejection. The first requires the use of SCNT techniques to generate autologous cells and eliminate the risk of graft rejection, an as-yet unsuccessful approach in human beings (Wilmut et al., 1997; Hentze 2006; Deb and Sarda 2008). Another option would be the induction of immune tolerance by genetically engineering hESC lines either by overexpressing genes that can suppress the immune system (Deb and Sarda, 2008), by deletion of MHC molecules or other immune effectors (Hentze et al., 2007; Mountford 2008), or by engineering hESC progeny to secrete locally immunosuppressive molecules (Murry and Keller, 2008). These methods are technically challenging and some would be clinically problematic, e.g., cells lacking MHC class I surface expression are targeted by natural killer cells (Hentze 2007; Deb and Sarda 2008). An alternative approach will be co-transplantation of the graft with haematopoietic stem cells generated from the same hESC line as used for tissue replacement, potentially inducing lifelong tolerance to the graft (Beilhack et al., 2003). This technique has been used in solid organ transplants where patients have previously received bone marrow transplantation, and these patients did not require immune suppression (Menendez et al., 2005; Priddle et al., 2006; Hentze et al., 2007). Yet another option would be to generate an hESC bank covering most histocompatibility antigen combinations to provide an acceptably well-matched graft for the majority of the population. It is estimated that several hundred thousand up to one million lines would be needed (Hyslop et al., 2005; Taylor et al., 2005; Rao and Auerbach, 2006). As only several hundreds lines have been generated so far, this is clearly going to be an impractical solution based on current technology and knowledge. Even with such banks immunosuppressive drugs would still be needed in most cases for most donor-patient combinations (Hentze et al., 2007; Deb and Sarda, 2008). Further, transplantation of allogeneic cells into immune-privileged sites such as the brain, may ameliorate the adverse effects of a MHC-

mismatch, although the protection might only be short term (Hentze et al., 2007; Deb and Sarda, 2008). Another straightforward approach useful for indications where cells do not have to undergo functional engraftment would be encapsulation of the graft with immune-inert material (Hentze et al., 2007). Although none of these approaches appears to be a complete solution for all indications, it is probable that a combination of different strategies will eventually facilitate successful transplantation of an allogeneic cell therapy product. The immunogenicity of each cell product however, must be tested at the appropriate site (Carpenter et al., 2009)

2.4.5 Epigenetic changes

Pluripotent cell lines can differ from each other in their genomic expression profiles through epigenetic regulations (Deb and Sarda, 2008). Epigenetic changes are associated with preimplantation embryos used for the derivation of the ESC lines and prolonged culture of pluripotent cells (Eggan et al., 2002; Draper et al., 2004; Allergrucci et al., 2007). In preimplantation embryos, epigenetic modifications such as DNA methylation and histone modifications are widely involved in the regulation of imprinted and non-imprinted genes. These events are often vulnerable to the external environment or culture conditions. It is possible that pluripotent cells can get rapidly reprogrammed into unpredictable genetic changes (Deb and Sarda, 2008). Most existing pluripotent cell lines have been established under different culture conditions, which may cause variations in epigenetic profiles over and above that inherited from *in vitro* production of the embryo (Wrenzycki and Niemann, 2003). A DNA methylation profile for hESC was determined, and a set of 25 sites from 23 genes was also identified that could be used to distinguish normal hESCs from differentiated cells (Bibikova et al., 2006). More studies on various existing pluripotent cell lines are required to identify the epigenetic markers for pluripotency. There is also a need for optimization of procedures that would minimise culture-induced genomic instability (Deb and Sarda, 2008). Periodic monitoring of epigenetic stability, by evaluating histone modification and DNA methylation, should be assessed after expansion of pluripotent cells in culture before the clinical use of pluripotent cell lines (Harrison et al., 2007; Spivakov and Fisher, 2007; Carpenter et al., 2009).

2.4.6 Chromosomal abnormalities

Selection and adaptation of hESCs is a poorly understood phenomenon (Deb and Sarda, 2008). Genomic instability of hESCs in long-term culture has been extensively investigated but less so with adult stem cells. Several reports indicate that hESCs acquire chromosomal abnormalities or karyotypic aberrations during prolonged culture in parallel with epigenetic changes (Mitalipova et al., 2005; Baker et al., 2007; Deb and Sarda, 2008; Carpenter et al., 2009). The acquisition of chromosomal abnormalities may be related to the laboratory manipulations of cells. More specifically, Draper et al., showed that trisomies 12 and 17 were observed when colonies were disaggregated to single cells, whereas manual passaging of cultures contributed to the long-term maintenance of normal karyotypes (Draper et al., 2004). Another report by Baker et al. demonstrates clear evidence for the

accumulation of specific chromosomal aberrations within several established hESC lines over time, indicating that hESCs become chromosomally abnormal and "culture adapted" in a reproducible, non-random nature over time. They also found a bias for gains of chromosomes 12, 17, and X, which is similar to the chromosomal change seen in breast cancer and testicular germ cell tumor seminomas and non-seminomas (Baker et al., 2007). Recently, two groups identified recurrent chromosomal abnormalities in hESCs maintained over long-term in culture, including amplification at 20q11.21, which is associated with oncogenic transformation (Lefort et al., 2008; Spits et al., 2008). Karyotypic changes often enhance the proliferative capacity of hESCs while shortening the population doubling time (Enver et al., 2005), and may result in a reduced tendency for apoptosis (Kristal et al., 2004) and a reduced capacity for differentiation, which is difficult to assess quantitatively. Therefore, reliable methods to monitor abnormal karyotype acquisition will be a crucial quality control assay for all cell lines to be used for research and especially for therapeutic purposes (Carpenter et al., 2009).

2.4.7 Tumorigenicity

Following transplantation, pluripotent stem cell based therapies involve the risk of tumor formation, such as teratomas comprising all three germ layers arising from an undifferentiated population of the transplanted cells or from an unstable cell product that may de-differentiate or transform the cells to produce a tumor (Carpenter et al., 2009). The immunosuppressive capacity of the adult stem cells may in some cases favor the growth of the tumor cells and, in contrast to hESCs, adult-sourced cell populations have already accumulated pro-oncogenic mutations, a situation that could be further exacerbated by *in vitro* culture selection of transformed cells with a higher growth rate (Hentze et al., 2006; Cousin et al., 2009). In the induction of pluripotent stem cells the introduction of c-Myc should be avoided for clinical application because c-Myc is associated with the development of tumors (Nakagawa et al., 2008). Another concern besides using oncogenes is that iPS cells contain retroviral integrations that could also increase the risk of tumorigenesis (Takahashi et al., 2007). Currently, the only way to ensure that teratomas do not form is to differentiate the stem cells in advance, enrich for the desired cell type, and screen for the presence of undifferentiated cells (Hentze et al., 2007).

As even a single undifferentiated cell in a differentiated transplant could carry tumor-forming potential, it seems that pluripotent cells should be at least partially differentiated before use in transplantation, but the extent to which they should be differentiated is a topic of much debate. Longer *in vitro* differentiation of pluripotent cells seems to reduce the risk of teratoma formation after transplantation (Brederlau et al., 2006). As the differentiation stage increases, however, the proliferative capacity of pluripotent cells is diminished such that mature cells are unable to proliferate. The majority of lineages have an identifiable precursor or progenitor cell population that can undergo extensive proliferation and expansion, but are no longer pluripotent. Thus, this partial differentiation stage may be an ideal population to use for cell therapy as the risks of teratoma and random differentiation are reduced and, as the cells still continue to proliferate, fewer cells would be required. Additionally, it may be that the *in vivo* environment would be far more effective in correctly inducing terminal differentiation than anything that can be reproduced *in vitro*. As

these precursor populations often closely resemble tissue-specific somatic stem cells that naturally repair tissue-damage, it may be that transplanted precursor populations would respond to *in vivo* signals and develop into the most appropriate cell type (Puceat and Ballis, 2007). Additionally, some injuries and diseases may require the replacement of a number of related lineages; for example, spinal cord injury results in the loss of neurons and astrocytes and also reduces myelination by oligodendrocytes (McDonald, 2004; Zhang et al., 2007). Therefore, a precursor population that could regenerate all the lineages may be a preferred option to single cell types. There is also the possibility, however, of tumors deriving from a progenitor cell that could stop the differentiation of the progenitor cells and proliferate *in vivo* (Puceat and Ballis, 2007). Such a scenario was reported for neuronal progenitors grafted in rat brain (Roy et al., 2006). Previously, teratomas were not observed in over 200 animals transplanted with an hESC-derived purified cardiomyocyte population (Laflamme et al, 2007), but more differentiated tumor-like growth might occur (Murry and Keller, 2008). A deeper understanding of the teratoma formation process should aid in the development of safer cell therapies and may help elucidate the basic principles of tumor initiation. Recent results suggest that continued expression of survivin, an anti-apoptotic oncofetal gene, upon differentiation *in vivo* may contribute to teratoma formation by hESCs (Blum et al., 2009).

The removal of all undifferentiated cells from the graft could be achieved by genetically engineering cells to express a suicide gene system. The expression of the suicide gene could be driven by the promoter of a gene such as Oct-4 that is only expressed in undifferentiated cells. This technology has been used in gene therapy systems (Doss et al., 2004). Alternatively, pluripotent cells can be genetically engineered so that a negative selection can be carried out based on a compound that is toxic to undifferentiated cells under certain culture conditions (Billon et al., 2002). Other methods include the expression of a genetic label such as green fluorescent protein (GFP) or a unique cell surface marker specific for either undifferentiated cells or differentiated progeny that enables cell isolation by fluorescence activated cell sorting (FACS; Strulovici et al., 2007; Murry and Keller, 2008). The principal drawback to genetic selection is the necessity of inserting a selection cassette into the host genome, which may increase the risk of tumorigenesis (Murry and Keller, 2008). While adequate technologies for screening and generating a pure population of differentiated stem cells are available, it is not yet known whether they are capable of eliminating each and every potential tumor forming pluripotent cell. Therefore, it is likely that a tumor assay for preclinical safety studies will be needed to test specific therapeutic stem cell-based formulations in the relevant environment (Hentze et al., 2007; Carpenter et al., 2009).

2.4.8 Other host factors

One major issue that needs to be considered before the administration of functional cell populations *in vivo*, is the type and number of cells delivered at the site of engraftment. In addition, transplanted cell death remains a major limitation for the treatment of many injuries and diseases. Current evidence suggests that cell-death pathways are initiated by multiple stresses associated with transplantation, including

ischemia, loss of matrix attachments, and inflammation (Murry and Keller, 2008). Most circumstances where regenerative therapy would be required would involve administration of the cells into a site of previous injury or disease where the local environment may be altered by the diseased state or by the presence of scarring or other lesions, and these intrinsic conditions could be hostile to the new graft (Nistor et al., 2005). Simply administration of stem cell-derived grafts into these zones would probably not result in any beneficial effect and other manipulations would be required to treat these established injuries before the graft could be effective (Deb and Sarda, 2008). Even if the environment is not hostile, it will still be necessary for the transplanted cells to integrate into the existing tissue and make the correct connections required to re-establish organ function (Mountford, 2008). Ideally, the presence of a clinical cell product should be traceable *in vivo*. Hence, sensitive techniques are required to measure the function as well as tracking the location and migration of the transplanted cells. There are a few techniques available for *in vivo* tracking of cells, including magnetic resonance imaging, radionuclide labeling and reporter gene imaging, but all of these techniques have their limitations and no method is currently sufficient to fulfill the more stringent requirements needed for human studies (Hentze et al., 2007).

3. Aims of the study

The general aim of the thesis was to develop and optimise defined and xeno-free culture conditions for stem cells supporting feasible proliferation while maintaining the characteristics and differentiation capacity of the different cell types and meeting the regulatory requirements set by the directives of the European Union for the clinical application of stem cells. In addition, the molecular mechanisms regulating the self-renewal and pluripotency of stem cells were studied. The specific aims of the thesis are outlined below.

1) To assess the applicability of an automated cell culturing, imaging, and analysis system for evaluating undifferentiated growth dynamics of hESCs maintained in different culture media.

2) To evaluate different xeno-free culture media for maintaining hESCs and to assess stem cell characteristics by comparing morphological characteristics, stem cell marker expression profiles and differentiation capacity.

3) To develop and optimise culture conditions for expanding stem cells according to the regulatory standards. To assess the applicability of the defined and xeno-free culture medium RegES in the derivation and expansion of new hESC lines and to evaluate stem cell characteristics by comparing the morphological characteristics, proliferation rate, stem cell marker expression profile, and differentiation capacity with those of hESC lines derived and expanded in conventional culture medium.

4) To evaluate the applicability of the defined and xeno-free culture medium RegES for the expansion of ASCs and to assess stem cell characteristics by comparing the morphological characteristics, proliferation rate, stem cell marker expression profile, and multipotentiality with those of ASCs expanded in allogeneic human serum.

5) To evaluate the applicability of the defined and xeno-free culture medium RegES for the expansion of iPS cells and to assess stem cell characteristics by comparing the morphological characteristics, stem cell marker expression profile, and differentiation capacity to those of iPS cells maintained in conventional culture medium.

6) To elucidate the stem cell characteristics as well as the molecular mechanisms regulating the self-renewal and pluripotency of hESCs under hypoxic conditions by assessing morphological characteristics, proliferation rate, stem cell marker expression profile, and signalling pathways.

4. Materials and methods

4.1 Ethical approval

The hESC lines used in these studies were derived at either the Karolinska Institutet (HS lines, Hovatta et al., 2003, Inzunza et al., 2005) or at the Regea – Institute for Regenerative Medicine (Regea lines, Rajala et al., 2010; Skottman unpublished data). The studies were conducted with the permission of the National Authority for Medicolegal Affairs to perform research with human embryos (Dnro 1426/32/300/05) and in accordance with the Karolinska Institutet Ethics Committee to derive and culture hESCs, with the Ethics Committee of the Pirkanmaa Hospital District to culture, characterise, and differentiate hESCs derived at Karolinska Institutet (R05051) and to derive, culture, characterise, and differentiate new hESC lines at Regea (R05116). Donated embryos were received from Turku and Tampere University Hospital IVF clinics (**IV**). An informed consent form was signed by both partners after receiving an oral and written description of the study. The donors did not receive financial compensation. Animal experiments were performed at the animal facilities of Karolinska University Hospital or at Turku Center for Disease Modeling and Department of Physiology, Institute of Biomedicine, University of Turku in accordance with the approval of the institution's Ethics Committee (**IV**). ASCs were isolated from adipose tissue samples collected from female donors undergoing elective surgical procedures at the Department of Plastic Surgery, Tampere University Hospital with the permission of the Ethics Committee of Pirkanmaa Hospital District (**IV**). Human iPS cells were derived and characterised at the University of Helsinki, with the permission of the Ethics Committee of the University of Helsinki (**IV**).

4.2 Cell culture (I-IV)

4.2.1 Derivation of human embryonic stem cell lines (IV)

Surplus poor-quality embryos that could not be used for infertility treatment were obtained as donations from couples undergoing IVF treatment at Tampere University Hospital. **In study IV,** the mechanical derivation of the hESC lines from blastocyst-stage embryos with a hardly visible ICM was performed using a specially made flexible metal needle and surgical knife (Ström et al., 2007). **In study IV,** derivation of the cell line Regea 06/015 was performed from an arrested embryo (around day 3-4) that did not undergo blastocyst formation, by removing the zona pellucida and plating the remaining cells on top of hFF cells.

4.2.2 Culture of human embryonic stem cells (I-IV)

In study I, hESCs were cultured in either conventional hES medium or in xeno-free X-vivo 10 medium. Conventional hES medium contained Knockout- Dulbecco's modified Eagle's medium (KO-DMEM) basal medium supplemented with 20% KO-SR, 2 mM Glutamax, 0.1 mM β-mercaptoethanol, 0.1 mM MEM with non-essential amino acids (NEAA), 1% penicillin-streptomycin, and 8 ng/ml bFGF. X-vivo 10 medium contained X-Vivo 10 basal medium and 0.12 ng/ml TGFβ1 otherwise supplemented as the hES medium without KO-SR. **In study II,** several xeno-free serum replacements and culture media were tested in the culture of hESCs. Conventional hES medium was used as a control medium. Xeno-free serum replacements Lipumin 10x, SerEx 10x, SR3, and Serum Substitute Supplement (SSS) were tested at 10% and 20% concentrations, and Plasmanate at 20% and 40% concentrations in KO-DMEM; X-vivo 10 medium (described above); X-Vivo 20 medium containing X-Vivo 20 basal medium; human serum medium containing KO-DMEM, 10% or 20% heat inactivated, sterile filtered human serum, 50 mg/L L-ascorbic acid 2-phosphate; and self-made TeSR1 medium (Ludwig et al., 2006). All were supplemented as the hES medium without KO-SR.

In study IV, hESC lines Regea 06/015, 07/046, and 08/013 were derived using a defined xeno-free culture medium RegES. All other hESC lines were derived using conventional hES medium. Xeno-free commercially available HEScGRO medium without any supplementation was compared to RegES medium for hESC culture. **In study III,** lipids and lipid derivatives were evaluated in the xeno-free RegES culture medium. KO-DMEM, DMEM low glucose, DMEM high glucose, and DMEM/F12 basal media, and the addition of IGF-II, IGF-I, or HRG-β1 was also evaluated in the xeno-free RegES culture medium. RegES culture media with an osmolarity of 260 mOsm, 290 mOsm, 320 mOsm, or 350 mOsm, were evaluated.

In studies I-IV, commercially available hFFs (CRL-2429, ATCC) cultured in Iscove's modified Dulbecco's medium (IMDM) with L-glutamine and 25 mM HEPES supplemented with 10% FBS and 1% penicillin-streptomycin were used for hESC culture. Confluent hFF monolayers were mitotically inactivated by irradiation (40 Gy) and plated at a density of 3.8×10^4 cells/cm^2. **In study IV,** hESC line Regea 06/015 was derived on hFF feeder cells (C-12300 PromoCell) cultured in IMDM with L-glutamine and 25 mM HEPES supplemented with 10% human serum, 0.1 mM NEAA, and 1% penicillin-streptomycin.

In studies I-IV, the hESCs were cultured in a humidified incubator (+37°C, 5% CO_2). The growth of hESCs was monitored under a microscope and culture media were changed six times per week. The hESCs were mechanically passaged every 6 to 7 days to new mitotically inactivated hFFs. **In study IV,** single-cell enzymatic passaging (SCED) was performed for hESCs using TrypLE Select and an 8 to 12 day splitting interval (Ellerström et al., 2007). The hESCs were frozen using RegES medium supplemented with 10% dimethyl sulfoxide (DMSO).

In study II, feeder cell free culture of hESCs was performed on 12-well plates (CellBIND Surface, Corning, NY), containing 10 µg/cm^2 human collagen IV, 0.2 µg/cm^2 human vitronectin, 5 µg/cm^2 human fibronectin, and 5 µg/cm^2 human laminin coating mixture. The feeder-free hESC cultures were passaged

mechanically every 7 to 10 days and plated onto new 12-well plates containing the coating mixture. For feeder-free culture experiments, hES medium and 20% human serum containing culture medium were supplemented with 80 ng/ml bFGF and the TeSR1 medium was supplemented with 100 ng/ml bFGF.

4.2.3 Culture of human induced pluripotent stem cells (IV)

Human iPS cell lines FiPS 5-7 and FiPS 6-14 were derived and characterised at the University of Helsinki. Human cDNAs of Oct-4, Sox2, Nanog, Klf4, and Lin-28, were amplified by direct PCR from hESC cDNA, and cloned into a pMXs retroviral vector. 293-GPG packaging cells were transfected with each pMXs-cDNA vector separately using Fugene HD. The next day, fresh MEF-CM medium was added. Viral supernatant was collected 3 times at days 4, 5, and 6 post-transfection, combined, and then filtered. The hFF cell line CCCD-1112Sk (ATCC) was plated on gelatin-coated petri dishes and incubated overnight. Cells were then infected with equal parts of hES medium and fresh virus-containing supernatant twice at 24-h intervals. At day 3, infected cells were harvested and re-seeded on a Mitomycin C-treated MEF layer. The following day, the medium was changed to hES medium. Twenty to 30 days post-transduction, ES-like colonies were picked, expanded, and characterised as previously described for hESCs (Mikkola et al., 2006). FiPS 5-7 was generated using Oct-4, Sox2, Nanog, and Lin-28 encoding retroviruses, while FiPS 6-14 was generated using the same factors plus Klf4. Human iPS cell lines were cultured as described for hESCs using hFF feeder cells, mechanical passaging, and hES or RegES culture media.

4.2.4 Culture of human adipose stem cells (IV)

Isolation of ASCs from adipose tissue samples was performed by mincing the adipose tissue manually into small fragments and digesting them with 1.5 mg/mL collagenase type I in a shaking water bath at 37 ^{o}C (Gimble and Guilak, 2003; Zuk et al., 2001). To separate the ASCs from the surrounding tissue, the digested tissue was centrifuged and filtered in sequential steps. The isolated cells were expanded in DMEM/F-12 1:1, supplemented with 1% GlutaMAX, 1% antibiotic/antimycotic (a/a; 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B) and 10% alloHS (alloHS medium). For testing defined xeno-free conditions, ASCs at passage 3-4 were transferred to RegES culture medium in flasks with pre-coated CELLstart. TrypLE Select was used to detach the ASCs from the culture plate.

4.2.5 Automation of imaging and analysis of human embryonic stem cells (I)

Human ESC lines were cultured and monitored using a time-lapse imaging system Cell-IQ® (Chipman Technologies Ltd, Tampere, Finland). The system comprised a thermal chamber in which two well plates, ranging in size from 6- to 96-well plates,

were set into an integrated plate holder and incubator gases were directly transferred into the plates. Two different gases could be piped onto a single plate. The chamber included a green LED light source below and microscopic phase-contrast optics (10 ×) above the well plates. A charge-coupled device camera was used for imaging. The system was connected to a computer through which the imaging was controlled with Cell-IQ® Imagen software. The plate holder could be moved in the xy-axis (± 1 μm) to enable complete control of plate movement. The areas of interest were selected and imaged in each well in single squares (500 × 670 μm) or in stitched grid squares of 2×2 up to 7×7 (ranging in size from 1000 × 1340 μm to 3500 × 4690 μm of imaged area). The system utilised a motorised z stage (± 0.4 μm) and a dynamic Z-stack (user defined) system, resulting in all-in-focus images. Single captured images were stored in separate folders as JPEG-files. These images could be opened and transformed into a movie format for further analysis. This allows for user-defined cell recognition programs utilizing machine vision technology to be built with analysis software.

We developed a new hESC colony area analysis method. The system recognises every pixel in each image and assigns the pixel to one of the user-defined classes. The colony area measurement classes were 1) background feeder layer, 2) undifferentiated area, and 3) differentiated area. The total analysed area in one image is usually 360448 pixels = (768-(2*32)) × (576-(2*32), wehre 768 is the image width and 576 is the image height. A 32 pixel-wide strip from the image edges was not analysed due to methodological limitations. The area analysis method results in total number of square pixels for each of the classes in each time-lapse image and that number can be converted to a micrometer scale.

The hESCs were cultured either in hES or X-Vivo 10 medium. The hESC colonies were mechanically divided and seeded as small aggregates onto 12-well plates (CellBIND surface) containing gamma-irradiated hFFs. On days 1 through 3 after passaging of hESCs, the plates were transferred to the monitoring equipment. Using the instrument's imaging software, the images from selected plate positions were recorded as grid images and stored in separate folders in JPEG format (Tarvainen et al., 2002). Each region of interest was monitored every 40 to 60 minutes. Colonies were monitored until day 7 after passage.

4.2.6 Culture of human embryonic stem cells under hypoxic versus normoxic conditions (III)

Human ESCs were cultured in hypoxic (5% O_2) and normoxic (21% O_2) conditions using Cell-IQ, an automated cell culturing system (Chipman Technologies, Tampere, Finland). Both conventional hES culture medium and serum- and xeno-free culture medium RegES were used. The cells were exposed to a 2-week culture period under normoxic or hypoxic conditions before analysis. Cells were manipulated under normoxic conditions and O_2 exposure of hESCs cultured under hypoxic conditions was kept to a minimum.

4.3 Differentiation of stem cells (IV)

4.3.1 Cardiomyocyte differentiation of human embryonic stem cells and induced pluripotent stem cells

Cardiomyocyte differentiation was carried out by co-culturing Regea 08/013 hESCs, maintained in RegES (p46) and in hES (p52) media, with mouse visceral endodermal-like (END-2) cells (Pekkanen-Mattila et al., 2009). Undifferentiated hESC colonies were dissected mechanically into aggregates containing a few hundred cells and placed on the top of plated END-2 cells in hES culture medium without KO-SR and bFGF. Differentiating cell colonies were monitored daily by microscopy and the medium was changed after 5 days of culturing. After 12 days, 10% KO-SR was added to the medium. Differentiation was performed in 12-well plates with 15 hESC colony pieces per well. The beating areas were dissociated by collagenase II treatment. The cells were plated on 0.1% gelatin coated cell culture plates in medium containing 7.5% FBS.

4.3.2 Neuronal differentiation of human embryonic stem cells and induced pluripotent stem cells

Human ESC (Regea 08/013) colonies were maintained in RegES (p53) and in hES (p58) culture media and mechanically dissociated into small clusters containing ~3000 cells. Clusters were transferred into 6-well ultra-low attachment plates (Nunc) and cultured as floating aggregates, e.g., neurospheres, for up to 20 weeks in neural differentiation medium consisting of 1:1 DMEM/F-12:Neurobasal media supplemented with 2 mM Glutamax, 1xB27, 1xN2, 25 U/ml penicillin-streptomycin, and 20 ng/ml bFGF (Hicks et al., 2009). Medium was changed 3 times/week and the spheres were mechanically passaged weekly.

4.3.3 Differentiation of adipose stem cells

ASCs at passage 3 were transferred in RegES medium to be analysed for their capacity to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages with ASCs expanded in alloHS medium as the reference. The osteogenic differentiation analyses were performed on ASCs seeded onto 12-well plates at a density of 2.5 x 10^3 cells/cm^2 in RegES medium supplemented with 150 µM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, and 100 nM dexamethasone in culture vessels pre-coated with CELLstart. The control cultures were maintained in RegES medium without supplements in pre-coated culture vessels. The cultures were maintained for 14 days and osteogenic differentiation was detected by alkaline phosphatase (ALP) activity staining.

Adipogenic differentiation was induced by culturing ASCs for 2 weeks in adipogenic RegES medium with an initial seeding density of 2 x 10^4 cells/cm^2 in culture vessels pre-coated with CELLstart. For adipogenic induction, RegES

medium supplemented with 33 µM biotin, 1 µM dexamethasone, 100 nM insulin, and 17 µM pantothenate was used while the control cultures were maintained in RegES without adipogenic supplements. The cultures were analysed using an Oil red O stain as an indicator of intracellular lipid accumulation.

Chondrogenic differentiation capacity analysis was assessed using a micromass culture technique (Denker et al., 1995; Zuk et al., 2001). Briefly, 1 x 10^5 cells were seeded onto a 24-well culture plates in a 10 µl volume, and allowed to adhere for 3 h in an incubator prior to the addition of chondrogenic RegES medium containing 1% ITS+1, 50 µM L-ascorbic acid 2-phosphate, 55 µM sodium pyruvate, and 23 µM L-proline. The control cultures were maintained in RegES without chondrogenic supplements. Chondrogenesis was confirmed at day 14 by Alcian blue staining.

4.4 Characterization of stem cells

4.4.1 Morphology (I-IV)

Cell morphology and growth was monitored using a stereomicroscope (Nikon SMZ800) or a phase-contrast microscope (Nikon T2000S). **In study I,** to cross-validate the growth data of the colonies obtained from the Cell-IQ analyses, all colonies were photographed using a Nikon Eclipse TE2000-S phase-contrast microscope and Eclipse Net software (version 1.20), enabling manual hESC colony area measurements.

In study III, lipids and lipid derivatives were evaluated with the RegES culture medium. General morphology and the size and thickness of the undifferentiated hESC colonies were evaluated before each passage based on visual inspection. The colonies were classified into four categories: excess differentiation, poor morphology; uneven colony edges, thin and/or small colonies, poor morphology; satisfying morphology, some colonies with uneven edges, colonies of medium thickness and size; and excellent morphology, even, thick and big colonies. In addition, the hESC colonies were classified into three categories; undifferentiated, partly differentiated, and differentiated (Figure 4). The number of each colony type was calculated before each passage. Later, a percentage value for each colony type of the total amount of colonies was calculated.

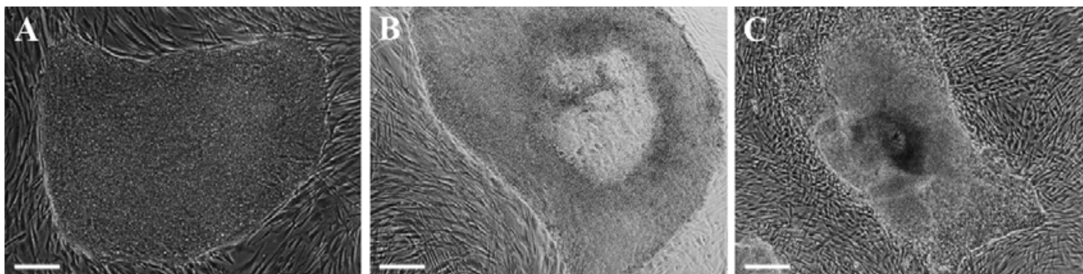

Figure 4. Classification of hESC colonies. Representative images of undifferentiated (A), partly differentiated (B) and differentiated (C) colony morphology. Scale bar 200 µm.

4.4.2 Immunocytochemistry (I-IV)

The cells were fixed in culture dishes with 4% paraformaldehyde (PFA) in phosphate-buffered saline (0.01 M PBS, pH 7.4) for 20 min at room temperature, followed by washing with PBS (2×5 min). The cells were permeabilised and blocked with 0.1% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS for 45 min at room temperature and then washed once with 0.1% Triton X-100, 1% BSA, and 1% normal donkey serum in PBS. The primary antibodies used for hESCs and iPS cells were specific for Nanog (1:200), Oct-4 (1:200), Sox2 (1:200), SSEA-4 (1:200), SSEA-1 (1:200), SSEA-3 (1:800), TRA-1-60 (1:200), and TRA-1-81 (1:200).

In study IV, the primary antibodies used for differentiated cardiomyocytes were anti-cardiac troponin T (cTnT; 1:2000) and anti-ventricular myosin heavy chain (vMHC; 1:100). Differentiated neural cells were immunostained after 9 weeks of differentiation. For immunocytochemical staining, neurospheres were plated on human laminin-coated wells in the absence of bFGF and fixed after allowing the cells to grow for 3 days. The primary antibodies used for differentiated neural cells were polyclonal rabbit anti-microtubule-associated protein-2 (MAP-2; 1:600) for neuronal cells and polyclonal sheep anti-glial fibrillary acidic protein (GFAP; 1:600) for astrocytes.

The cells were incubated with primary antibody solution overnight at 4°C. The cells were washed (3×5 min) with 1% BSA in PBS and probed with secondary antibodies: rhodamine red-conjugated anti-mouse at 1:400 and Alexa Fluor-488 at 1:800 or Alexa Fluor-568 at 1:400 conjugated to anti-goat or anti-mouse, and FITC conjugated to anti-rabbit or anti-sheep. Cells 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 (DAPI). The labelled cells were photographed with an Olympus IX51 phase contrast microscope with fluorescence optics and an Olympus DP30BW camera.

In study IV, the *in vitro* osteogenic differentiation capacity was determined 14 days after the initiation of differentiation by ALP staining as described previously (Haimi et al., 2008; Haimi et al., 2009 and Lindroos et al., 2009). The cell cultures were fixed with a 4% PFA solution and stained with the leukocyte ALP kit according to the Sigma Procedure No. 86 (#86R-1KT). ALP staining was confirmed as a purple staining visualised by microscopy.

The adipogenic differentiation culture was maintained for 14 days and subsequently fixed with 4% PFA, pretreated with 60% isopropanol, and stained with a 0.5% Oil red O solution in 60% isopropanol. After fixation and staining, the wells were rinsed with distilled water and visualised by microscopy. Adipocytes were identified as cells with red-stained lipid vesicles.

The chondrogenic differentiation cultures were maintained for 14 days and were subsequently fixed with 4% PFA, and stained with 1% Alcian blue stain. Cells stained with Alcian blue verified the presence of sulphated proteoglycans within the chondrogenic cell pellet.

4.4.3 Flow cytometry (I-IV)

Human ESCs and iPS cells were analysed by flow cytometry using antibodies against SSEA-1, SSEA-4 (**I,II**), PE-conjugated SSEA-4 (**III,IV**), Oct-4-PE, and TRA-1-81-FITC. For the SSEA-1 and SSEA-4 primary antibodies, a PE-conjugated antibody was used as the secondary antibody.

In study IV, ASCs cultured for 1 week in RegES medium were analysed by flow cytometry using monoclonal antibodies against CD90- APC, CD45-FITC, CD34-APC, human leucocyte antigen class one (HLA-ABC)-PE, HLA-DR-PE, and CD105-PE. **In study IV,** hESC samples cultured in hES and RegES media were from 7 day old colonies, iPS cell samples cultured in hES medium from 6-day-old colonies, and samples cultured in RegES medium from 7-day-old (5-7) or 8-day-old colonies (6-14). Both undifferentiated and differentiated cells present in the culture dish were included in the analyses. **In studies I-IV,** Alexa Fluor 488 and PE-conjugated secondary antibodies were used as isotype controls. The samples were analysed by FACS (FACSAria®, BD Biosciences). The acquisition was set for 10 000 events per sample. The data were analysed using FACSDiva Software version 4.1.2.

4.4.4 Cell proliferation assay (III, IV)

Cell proliferation of the hESC lines was determined using a colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation during DNA synthesis. The enzyme linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. The hESC colonies at day 5 were labelled with BrdU-labelling solution overnight at 37°C. The hESCs (10^5 cells/well) were added to a 96-well plate with 8 replicates per cell line. Cells that were not labelled with BrdU were used as a background control. Absorbance of the samples was measured at 450nm using a Viktor 1429 Multilabel Counter.

In study IV, the cell viability and proliferation activity of the ASCs were assessed using a PreMix WST-1 Cell Proliferation Assay System. The ASCs (n=7 donor cell samples, with 4 replicates per cell line) at passage 3 were seeded on 48-well plates at a density of 4500 cells/cm^2 in RegES medium, and the proliferation was determined at 1, 4, 7, and 11 days. Briefly, the cell culture medium was removed and DPBS and PreMix WST-1 were added at a ratio of 10:1. The well plate was incubated for 4 hours in 37°C and the cell proliferation activity was measured at 450 nm using a Viktor 1429 Multilabel Counter.

4.4.5 RNA isolation and reverse transcription (II-IV)

Total RNA was isolated using an RNeasy Plus Mini Kit according to the manufacturer's instructions. The concentration and quality of isolated RNA was determined using a ND-1000 Spectrophotometer. Complementary DNA was synthesised from 50 ng of total RNA using a Sensiscript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

4.4.6 RT-PCR (II, III)

The expression of ectoderm (PAX-6, Sox1), endoderm (α-fetoprotein (AFP), Sox17), and mesoderm (α-cardiac actin, brachyury/T) development in EBs was confirmed using the primers listed in Table 3.˜ β-actin was used as a housekeeping control. Negative controls contained sterilised water instead of the cDNA template. The RT-PCR reactions were performed in an Eppendorf Mastercycler as follows: denaturation at 95°C for 3 min; 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 a final extension at 72°C for 5 min. The PCR products were analysed by electrophoresis on a 1.5% agarose gel containing 0.4 μg/ml ethidium bromide and 50 bp DNA standard.

Table 3. Complete list of primers used for RT-PCR in all publications.

Name	Primer sequence	Size (bp)	Study
Oct-4	*F* 5´ CGTGAAGCTGGAGAAGGAGAAGCTG 3´ *R* 5´ AAGGGCCGCAGCTTACACATGTTC 3´	245	IV
Nanog	F5´ TGCAAATGTCTTCTGCTGAGAT 3' R5' GTTCAGGATGTTGGAGAGTTC 3'	286	IV
AFP	*F* 5´ GCTGGATTGTCTGCAGGATGGGGAA 3´ *R* 5´ TCCCCTGAAGAAAATTGGTTAAAAT 3´	216	II,IV
Brachyury/T	*F* 5´ GCTTCAAGGAGCTCACCAAT 3´ *R* 5´ CACCGCTATGAACTGGGTCT 3´	425	IV
Musashi	*F* 5´ AGCTTCCCTCTCCCTCATTC 3´ *R* 5´ GAGACACCGGAGGATGGTAA 3´	161	IV
Nestin	F 5´ CAGCTGGCGCACCTCAAGATG 3´ R 5´ AGGGAAGTTGGGCTCAGGACTGG	208	IV
PAX-6	*F* 5´ AACAGACACAGCCCTCACAAACA 3´ *R* 5´ CGGGAACTTGAACTGGAACTGAC 3´	174	IV
OTX2	F5' CGCCTTACGCAGTCAATGGG 3' R5' CGGGAAGCTGGTGATGCATAG 3'	641	IV
MAP-2	*F* 5´ AATAGACCTAAGCCATGTGACATCC 3´ *R* 5´ AGAACCAACTTTAGCTTGGGCC 3´	132	IV
NF-68	*F* 5´ GAGTGAAATGGCACGATACCTA 3´ *R* 5´ TTTCCTCTCCTTCTTCACCTTC 3´	473	II,IV
GFAP	*F* 5´ GCTCGATCAACTCACCGCCAACA 3´ *R* 5´ GGGCAGCAGCGTCTGTCAGGTC 3´	207	IV
Olig1	*F* 5´ TTGCATCCAGTGTTCCCGATTTAC 3´ *R* 5´ TGCCAGTTAAATTCGGCTACTACC 3´	389	IV
GAPDH	*F* 5´ AGCCACATCGCTCAGACACC 3´ *R* 5´ GTACTCAGCGGCCAGCATCG 3´	302	II,IV
β-Actin	F5' GTCTTCCCCTCCATCGTG 3' R5' GGGGTGTTGAAGGTCTCAAA 3'	302	IV
α-cardiactin	F5' GGAGTTATGGTGGGTATGGGTC 3' R5' AGTGGTGACAAAGGAGTAGCCA 3'	486	II,IV
AFP	F5' AGAACCTGTCACAAGCTGTG 3' R5' GACAGCAAGCTGAGGATGTC 3'	672	IV
Sox17	F5' CGCACGGAATTTGAACAGTA 3' R5' CACACGTCAGGATAGTTGCAG 3'	166	IV
Sox1	F5' AAAGTCAAAACGAGGCGAGA 3' R5' AAGTGCTTGGACCTGCCTTA 3'	158	IV

In study IV, RT-PCR was performed for neural cells (≥10 spheres/sample). The RT-PCR primer set contained GAPDH as a housekeeping control; Oct-4 and Nanog for undifferentiated hESCs; α-fetoprotein for endodermal lineage; brachyury/T for mesodermal lineage; Musashi, Nestin, and PAX-6 for neuroectodermal cells; MAP-2, NF-68, and OTX2 for neuronal cells; GFAP for astrocytes; and Olig1 for oligodendrocyte precursor cells (listed in Table 3). The RT-PCR reactions were performed in an Eppendorf Mastercycler with an initializing step at 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were analysed by electrophoresis on a 1.5% agarose gel containing 0.4 µg/ml ethidium bromide and 50 bp DNA standard.

4.4.7 Quantitative PCR (III-IV)

In studies III and IV, both undifferentiated and differentiated hESCs and iPS cells in the culture plate were included in the quantitative RT-PCR analyses. Quantitative RT-PCR was performed with the Applied Biosystems Gene Expression Assays listed in Table 4. All samples and the no-template controls were analysed in triplicate. Quantitative RT-PCR was performed with an Applied Biosystems 7300 Real-Time PCR system using the following conditions: 40 cycles of 50°C for 2 min, 95°C for 10 min, and 95°C for 15 s, followed by 60°C for 1 min. The data were analysed with a 7300 System SDS Software. The cycle threshold (Ct) values were determined for every reaction. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All data were normalised to the expression of the housekeeping gene GAPDH.

Table 4. Complete list of primers for undifferentiated hESCs and iPS cells for qRT-PCR in all publications.

Name	Assay ID	Size (bp)	Study
GAPDH	Hs99999905_m1	122	III, IV
Nanog	Hs2387400_g1	109	III, IV
Oct-4	Hs00999632_g1	77	III, IV
DNMT3B	Hs01003405_m1	80	III, IV
TDGF1	Hs023339496_m1	102	III, IV
GDF3	Hs00220998_m1	65	III, IV
GABRB3	Hs01115771_m1	72	III, IV

In study III, the molecular mechanisms activated by low O_2 tension were investigated using a customised PathwayFinder™ RT²Profiler™ real-time PCR Array (CAPH-0616, SABiosciences) including genes from the different signalling pathways listed in Table 5. cDNA was synthesised from 1.0 µg of total RNA using the RT^2 First Strand Kit according to the manufacturer's instructions. Quantitative RT-PCR was performed with an Applied Biosystems 7300 Real-Time PCR system using the following conditions: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. The data were analysed with a 7300 System SDS Software. The Ct values were determined for every reaction. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method. All data were normalised to the expression of the housekeeping genes.

Table 5. Signal transduction and growth factor pathways analysed in publication **III**.

Pathway	**Gene**
Mitogenic	EGR1, FOS, JUN
Hedgehog	BMP2, BMP4, EN1, FOXA2, PTCH1, WNT1, WNT2
Wnt	CCND1, JUN, LEF1, MYC, PPARG, TCF7, VEGFA, WISP1
TGF-β	CDKN1A, CDKN1B, CDKN2A, CDKN2B
Survival pathway:	
PI3 kinase/Akt	BCL2, CCND1, FN1, JUN, MMP7, MYC
Jak/Src	BCL2, BCL2L1
NFκB	BCL2A1, BIRC2, BIRC3, NAIP, TERT
P53	BAX, CDKN1A, Fas, GADD45A, IGFBP3, MDM2, TP53I3
CREB	CYP19A1, EGR1, FOS
Jak-Stat	CXCL9, IL4, IL4R, IRF1, MMP10, NOS2A
Retinoic acid	EN1, HOXA1, RPB1
Insulin	CEBPB, FASN, GYS1, HK2, LEP
Insulin signalling	ELK1, FOS, FOXO1, JUN
Phospholipase C	BCL2, EGR1, FOS, ICAM1, JUN, NOS2A, PTGS2, VCAM1
Calcium and PKC	CSF2, FOS, IL2, JUN, MYC, ODC1, PRKCA, PRKCE, TFRC
EGF	ELK1, FOS, JUN, STAT1, STAT3, STAT5A
IGF-1	ELK1, FOS, JUN
PDGF	ELK1, FOS, JUN, STAT1, STAT3, STAT5A
G-protein coupled R	CREB1, ELK1, FOS, JUN, NFATC1
Notch	CREBBP, HDAC1
MAPK:	
Erk1/Erk2	ELK1, MYC, STAT3
P38 signalling	ATF2, CREB1, ELK1, MAX, MEF2A, MYC, STAT1
Hypoxia signalling:	
Response to stress	IL1A, IL6, NOS2A, NOTCH1, RARA
Transcription factors and regulators	CREBBP, RARA, HIF1A-HIF3A, PPARA, NOTCH1
Signal transduction	CREBBP, HIF1A-HIF3A, IGFBP1, IL1A, IL6, LEP, RARA, VEGFA
Cell cycle	BAX, HK2, IGF2, IL1A, VEGFA
Cell proliferation	DCTN2, IGF2, IL1A, IL6, MT3, NPY, RARA, VEGFA
Growth factors	IGF2, IGFBP1, IL1A, IL6, VEGFA

4.4.8 Karyotyping (IV)

Karyotype analysis was performed using the G-banding technique by Medix Laboratories Inc, Helsinki, Finland. In brief, hESCs were treated with colchicine for 2 hours, and cells in metaphase were analysed using conventional light microscopy (Olympus, BX 50). Karyotypes were determined using IKAROS-software designed for chromosome analysis (MetaSystems). A total of 20 cells in metaphase were analysed for each cell line.

4.4.9 Analysis of pluripotency *in vitro* (II, IV)

The EBs formed by mechanically dissecting the hESC and iPS cell colonies were cultured without feeder cells in TeSR1 **(II)**, RegES, or hES **(IV)** culture medium without bFGF for 4 weeks before RNA isolation. The medium was changed every 2 to 3 days. RNA isolation, reverse transcription, and RT-PCR from EBs were performed as described above.

4.4.10 Analysis of pluripotency *in vivo* (IV)

The *in vivo* pluripotency of the hESC line Regea 06/015, Regea 07/046, Regea 08/013 and Regea 06/040 was tested as previously described (Inzunza et al., 2004; Inzunza et al., 2005). In brief, the cells were harvested from the culture plates using dispase or TrypLe Select and mechanical treatment. Five colonies (10^3 to 10^4 hESC) were washed twice in PBS and subsequently implanted beneath the testicular capsule of a young (6-8 week-old) SCID/beige male mouse (C.B.-17/GbmsTac-scid-bgDF N7, M&B, Ry, Denmark) or in the case of Regea 08/013, a male nude mouse (Hsd; Athymic Nude-Foxn1^{nu}, Harlan). Three animals were used for each cell line. Teratoma growth was determined by palpation once per week, and the mice were killed by cervical dislocation 8 to 9 weeks after implantation. Immunohistochemical studies of Regea 08/013 cell line teratomas were performed on paraffin sections. The dewaxed and rehydrated sections were treated in a pressure cooker in 1 mM EDTA, pH 8, (Desmin and HNF3β) or 10 mM sodium citrate buffer, pH 6, (NCAM) for 5 minutes to reveal antigenic sites, cooled at room temperature for 2 hours, and rinsed in PBS. After rinsing in PBS the sections were incubated with the following primary antibodies: rabbit anti-desmin, mouse anti-HNF3β, or rabbit anti-NCAM. Primary antibodies were diluted (1:1000, 1:3000, and 1:800 respectively) in PBS containing 0.05% Tween® and incubated overnight at +4°C. The next day, after several rinses with PBS, endogenous peroxidase was inactivated with 1% hydrogen peroxide in PBS for 20 minutes. After rinsing several times with PBS, the sections were incubated with secondary antibodies (conjugated to horseradish peroxidase) for 30 minutes at room temperature. Slides were rinsed with PBS, and the color was developed with diaminobenzidine substrate. Sections were lightly counterstained with Mayer's haematoxylin, dehydrated, and mounted.

4.5 Statistical analysis (I, III, IV)

In study I, statistical analyses were performed with SPSS for Windows software (V9.0) using a nonparametric test (the Kruskall-Wallis test) followed by *post hoc* analysis (the Mann-Whitney *U*-test). *P*-values less than 0.05 were considered significant. Differences between the hESC lines and culture conditions were tested using Wilcoxon's signed rank test.

In study III, the values of surface marker expression and proliferation of hESCs, expressed as mean ± SD, were compared between the hypoxic and normoxic conditions using Student's t test. In the proliferation analysis, comparisons were made between hypoxic and normoxic conditions for all cell lines, cell lines cultured only in the hES medium, and cell lines cultured only in the RegES medium. P values less than 0.05 were considered significant. The data were analysed using SPSS for Windows (v 14.0).

In study IV, mixed models ANOVA was used for the proliferation data analysis of ASCs between the different culture media compositions within each separate experiment setup to account for the correlation of multiple measurements within donors. The Mann-Whitney U test was applied to assess the surface marker expression of ASCs between different culture conditions. The surface marker expression of ASCs was analysed using the Wilcoxon signed-rank statistic, which was used for pairwise comparison between the different culturing conditions. A p value of less than 0.05 was considered statistically significant. The analyses were performed using SPSS for Windows (v 13.0).

5. Results

5.1 Automation of imaging and analysis (I)

In the present study, an automated culturing, imaging, and analysis system, Cell-IQ®, was used to study the growth dynamics of hESCs during one passaging interval. We developed an hESC colony area analysis method based on the area measurement of the background feeder layer, undifferentiated colony area, and differentiated colony area. The average size of the HS237 and HS293 colonies, excluding totally differentiated and non-growing hESC colonies, at day 6 was 0.99 mm^2 (± 0.61, *n* = 24), with 91% undifferentiated cells and 9% differentiated cells. To validate the results of the automated analysis, flow cytometry analysis and immunocytochemical staining analysis were performed. Flow cytometry analysis of the pooled HS237 colonies (n = 143), with a total of 3.0 million cells at day 7, revealed that an average-sized colony of 1.04 mm^2 contained ~21,000 cells (n = 29). Based on the flow cytometry analysis, 81% of the HS237 cells were positive for SSEA-4, which was consistent with the automated area protocol result, indicating that 86.9% of the HS237 colony area (n = 29) was undifferentiated. Immunocytochemical staining of the colonies showed that these colonies were positive for Nanog and that SSEA-1 was expressed in only a small proportion (3%) of the cells.

After validating the automated analysis protocol, we further examined the growth parameters of the HS237 and HS293 cell lines. The average colony size did not differ between HS237 (0.79 ± 0.32 mm^2) and HS293 (1.20 ± 0.80 mm^2) cell lines at day 6. The growth rates of the undifferentiated areas (from days 5 to 6) of HS237 (215 ± 94 μm^2/min) and HS293 (328 ± 187μm^2/min) were also similar. HS293 colonies contained more undifferentiated areas than HS237 colonies ($P < 0.01$).

The automated culturing, imaging and analysis system was also used to evaluate two different culture media, conventional hES culture medium and an X-vivo 10-based, xeno-free culture medium. Both HS237 and HS293 cell lines grew better in hES medium than in X-vivo 10 medium. Average colony size for both HS237 and HS293 was smaller in the X-vivo 10 medium than in the hES medium (0.44 ± 0.31 vs. 0.99 mm^2 ± 0.61, $P < 0.01$), and the undifferentiated colony area was smaller in colonies cultured in the X-vivo 10 medium (64%) than in the hES medium (91%, P <0.01).

5.2 Evaluation of xeno-free media formulations (II)

5.2.1 Feeder cell-dependent culture of human embryonic stem cells (II)

In this study, we evaluated nine commercially available or published xeno-free medium formulations (Lipumin™, SerEx, SSS, SR3, X-Vivo10, X-Vivo 20, Plasmanate, TeSR1, and human serum) for their ability to maintain hESC proliferation and self-renewal. Conventional hES medium was used as a control in all experiments.

The hESC lines HS181 and HS237 cultured in the xeno-free media formulations either differentiated or their proliferation decreased substantially. The results were consistent for both hESC lines and across repeated experiments. Differentiation of the hESCs began during the adaptation process with all test media except TeSR1 and human serum media, as indicated by the change in colony morphology. More specifically, the differentiated colonies became thinner, and lost their angular shape and sharp edges. Differentiation was confirmed by immunocytochemical staining analysis; hESC colonies cultured with the test media showed an increased expression of a marker characteristic of differentiated hESCs (SSEA-1), but lacked the expression of a marker characteristic of undifferentiated hESCs (Nanog). In the presence of X-Vivo 10, SSS, SerEx, and TeSR1, colony growth was reduced. With X-Vivo 10, X-Vivo 20, SR3, and Lipumin™, hFF morphology was spherical and underwent premature detachment. Except in TeSR1 and human serum media, the hESCs were all differentiated when the adaptation process was complete and it was impossible to passage the colonies further. TeSR1 medium was unable to maintain hESC self-renewal beyond 7 passages. An *in vitro* differentiation analysis was performed for the hESCs cultured in TeSR1 medium. Despite the detection of ectoderm (NF-68) and mesoderm (α-cardiac actin) -specific markers, an endoderm (α-fetoprotein) -specific marker was not detected, indicating defective pluripotency of hESCs cultured in TeSR1 medium.

The hESCs cultured in 10% and 20% human serum medium underwent excessive differentiation during the first passages of the adaptation phases, which was observed as a change in the hESC colony morphology. More specifically, the colonies were thinner and lost their angular shape and defined borders. Human serum medium-10% was able to maintain the growth of undifferentiated hESCs for 9 passages. At the end of the passage 10, all colonies had completely differentiated, which was confirmed by immunocytochemical staining with Nanog and SSEA-1. As the colonies in 20% human serum medium were passaged further, they began to regain their undifferentiated morphology. Moreover, at passage 11 the colonies had mainly an undifferentiated morphology, although they were notably thinner than the hESCs cultured in hES medium. At passage 11, the hESCs cultured in 20% human serum medium were stained with Nanog, Oct-4, SSEA-4, and SSEA-1 immunofluorescent antibodies. Some of the colonies did not express hESC-specific markers or SSEA-1, and hESCs cultured in hES medium strongly expressed Nanog and did not express SSEA-1. Flow cytometry analysis demonstrated that only 35% of the hESCs cultured in 20% human serum medium and 80% of the hESCs

cultured in hES medium were positive for SSEA-4. Despite the excessive differentiation at the beginning of the experiment, our results indicate that a population of cells was able to adapt to the culture conditions with human serum.

5.2.2 Feeder cell-free culture of human embryonic stem cells (II)

Control hES, TeSR1, and 20% human serum medium were used to evaluate the self-renewal of hESC line HS237 in feeder cell-free culture conditions using the previously described xeno-free ECM coating mixture (Ludwig et al., 2006). From the beginning of the experiment, it was obvious that the attachment of hESCs to the ECM mixture was defective. Specifically, of the hESC colony pieces plated with each medium, only 30% attached in TeSR1 medium, 55% in 20% human serum medium, and 68% in hES medium. In addition, of the attached colony pieces, only 30% formed colonies in TeSR1 medium, while 75% formed colonies in 20% human serum medium and hES medium. After the second passaging, only occasional colony formation was observed in hES medium and in 20% human serum medium while no colony formation was observed in TeSR1 medium, indicating that none of these medium formulations was able to maintain hESC self-renewal in the absence of a feeder cell layer.

5.3 Development and optimization of xeno-free medium formulation (III)

We developed a basic defined and xeno-free serum replacement formulation (RegES) based on human serum albumin (HSA), transferrin, amino acids, vitamins, antioxidants, trace minerals, and growth factors including bFGF and insulin. The initial evaluation of the xeno-free RegES medium revealed that the colonies were thinner and the growth of the hESCs was slower than in hESC lines cultured in the conventional hES medium. A new hESC line, Regea 06/015, was successfully derived by using the first version of the xeno-free RegES medium and hFFs cultured in human serum medium. Subsequently, the growth of the Regea 06/015 cell line decreased and at passage 7 it was transferred to hES medium. The cell line was karyotypically normal and exhibited normal hESC characteristics, including stem cell marker expression and pluripotency as determined by EB analysis and teratoma formation.

5.3.1 Evaluation of basal media, glucose concentration, and osmolarity

To evaluate different basal media we tested DMEM/F12, DMEM low D-glucose (1 g/L), DMEM high D-glucose (4.5 g/L) and KO-DMEM basal media formulations with the hESC line HS401 for 5 passages. DMEM/F12 and DMEM low D-glucose resulted in excess differentiation of hESCs, whereas only minor differences in the proliferation and colony morphology were observed with KO-DMEM and DMEM

high D-glucose. In the presence of DMEM/F12 and both DMEM formulations, the hFFs exhibited an altered spherical morphology, whereas in KO-DMEM the hFFs exhibited the normal elongated morphology. Of the different basal media, KO-DMEM had the best performance with the RegES concentrate, indicating that a high D-glucose concentration and reduced osmolarity of KO-DMEM were more suitable for hESC culture.

To further optimise the formulation, we evaluated an osmolarity in the range of 260 to 350 mOsm in the hESC culture for 5 passages. An osmolarity of 260 mOsm induced the formation of small uneven colonies and although the morphology of the colonies was improved at an osmolarity of 290 mOsm, the colony size remained small. An osmolarity of 350 mOsm clearly restricted the growth of the colonies. The best performance was obtained with an osmolarity of 320 mOsm resulting in normal colony morphology with defined borders and adequate size.

5.3.2 Effect of specific lipids and lipid derivatives

As AlbuMAX, a lipid-rich BSA, is the only non-chemically-defined component of KO-SR, we studied whether the addition of lipids or lipid derivatives could further enhance the performance of the xeno-free formulation. To assess the general morphology, as well as the size and thickness of the undifferentiated colonies, we performed visual evaluation of the colonies before each passage. The results indicated that conjugated linoleic acid (CLA), eicosapentaenoic acid (EPA), palmitoleic acid, linoleic acid, linoleic-oleic-arachidonic acid mix, and especially retinol improved the morphology of the undifferentiated colonies in both hES and RegES culture media. In addition, myristic acid, alfa-linoleic acid, docosahexaenoic acid, S1P, and PGE_2 resulted in poor morphology and induced rapid differentiation in both types of culture media.

Besides evaluating the morphology of the undifferentiated colonies, the hESC colonies were classified into three categories; undifferentiated, partially differentiated, and differentiated. The number of each colony type was counted before each passage, enabling the calculation of a percentage value for each colony type of the total amount of colonies. In RegES culture medium, the number of undifferentiated colonies increased and the number of differentiated colonies decreased in the presence of CLA, EPA, and stearic acid compared with colonies cultured in the control hES medium. In addition, the number of undifferentiated colonies increased in the presence of retinol, a linoleic-oleic-arachidonic acid mix, DL-isoproterenol, palmitoleic acid, and linoleic acid compared with the colonies cultured in the Albumax-RegES medium.

According to the morphology and the level of different colony types, retinol, CLA, and EPA improved colony morphology and decreased the number of differentiated colonies in both culture media.

5.3.3 Retinol and Activin A increase the proliferation and expression of stem cell markers

The effects of retinol were further evaluated in the xeno-free RegES medium. An ELISA-based cell proliferation assay demonstrated that retinol (0.5 μM, 2.0 μM, and 3.5 μM) stimulated the proliferation rate of hESCs by 5%, 34%, and 37%, respectively. In addition, it was noticed that the colony size increased in the presence of retinol. Quantitative RT-PCR analysis on hESCs cultured for 10 passages with or without retinol demonstrated that while a less than 2-fold increase in the gene expression of Oct-4, GDF3, DNMT3B, and TDGF1 was detected, retinol (0.5 μM, 2.0 μM, and 3.5 μM) increased the expression of Nanog, by 4, 23, and 25-fold, respectively. Furthermore, flow cytometry analysis of hESCs cultured for 10 passages with and without 2.0 μM retinol showed that retinol induced the expression of hESC-specific markers SSEA-4 from 38% to 63% and TRA-1-81 from 37% to 54%. To assess whether increased expression of the Nanog mRNA results in protein synthesis, immunocytochemical analysis was performed on hESCs cultured without and with retinol (0.5 μM, 2.0 μM, and 3.5 μM). The analysis revealed enhanced staining for Nanog in the presence of retinol (Figure 5).

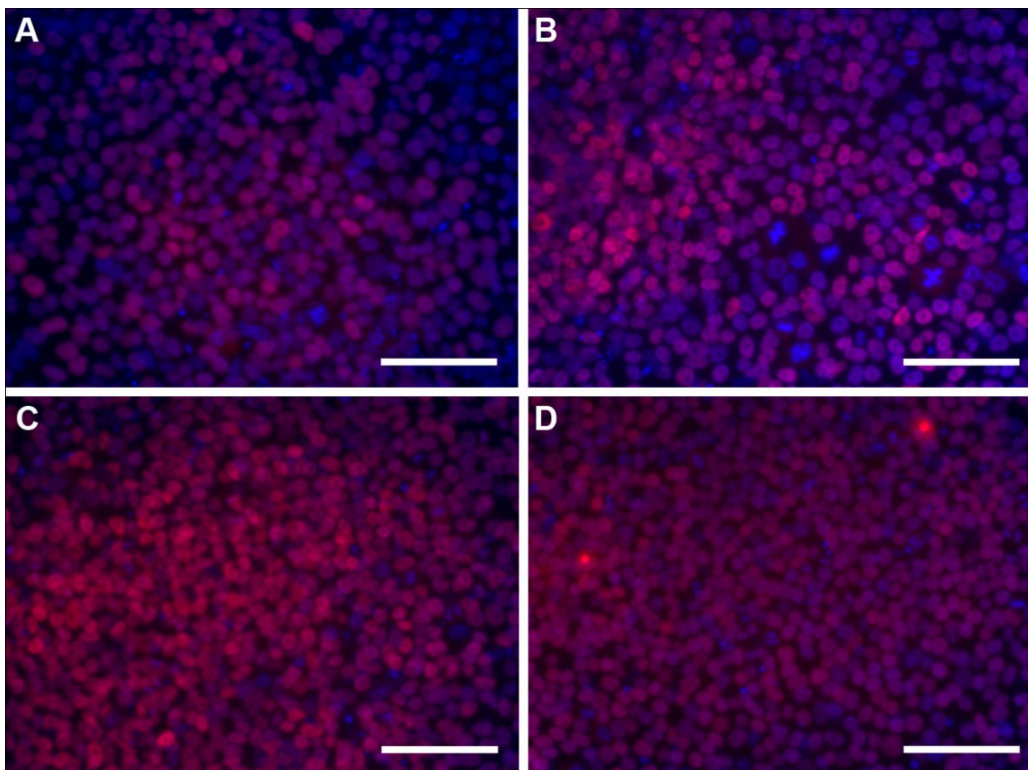

Figure 5. Fluorescence microscopy images of hESCs cultured without (A) and with 0.5 μM (B), 2.0 μM (C), and 3.5 μM (D) retinol for 12 passages showing the expression of Nanog (red) and DAPI (blue). Scale bar: 200 μm.

Growth factor Activin A was also evaluated in the xeno-free RegES medium. An ELISA-based cell proliferation assay performed for hESCs cultured with or without Activin A demonstrated that Activin A (5 ng/ml and 10 ng/ml) stimulated the proliferation rate of hESCs by 68% and 63%, respectively (Figure 6A). Flow cytometry analysis of hESCs cultured with and without Activin A (5 and 10 ng/ml) showed that Activin A increased the expression of the hESC-specific markers SSEA-4 and TRA-1-81 (Figure 6B).

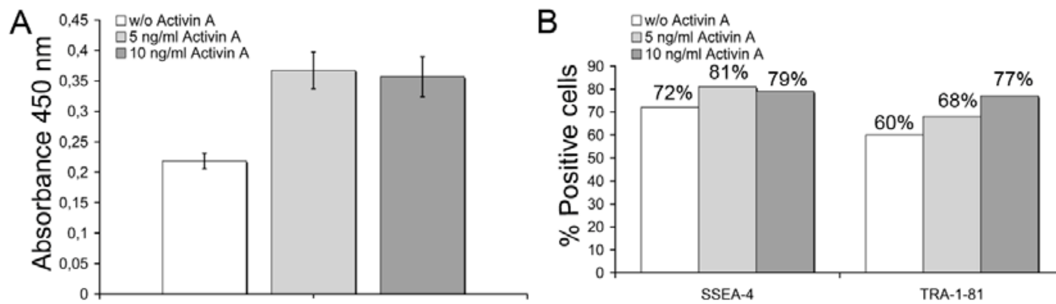

Figure 6. Activin A induced proliferation and stem cell marker expression. A) Cell proliferation analysis of hESCs cultured without and with 5 and 10 ng/ml Activin A for two passages. B) FACS analysis of hESCs cultured without and with 5 and 10 ng/ml Activin A for two passages.

5.3.4 Insulin induces the expression of stem cell markers

To assess whether insulin is an important component of the xeno-free medium formulation, we tested both IGF-I and IGF-II as well as a combination described by Wang et al., 2007 containing 10 ng/ml Activin A, 200 ng/ml IGF-I, and 10 ng/ml HRG-β1 for the ability to maintain the proliferation and undifferentiated growth of hESCs without insulin. Without insulin and in the presence of IGF-I, IGF-II, and the combination of 10 ng/ml Activin A, 200 ng/ml IGF-I, and 10 ng/ml HRG-β1 less compact and thinner colonies with less clearly defined borders, as well as increased differentiation of hESCs was detected. The cultures could not be maintained beyond 5 passages due to difficulties in the mechanical passaging of the thin and fragile colonies. When a combination of Activin A, IGF-I, and HRG-β1 was evaluated in the RegES formulation containing insulin, the colonies exhibited better morphology and proliferation, but were thinner compared to colonies cultured in the RegES formulation containing only insulin. Quantitative RT-PCR of hESCs cultured with or without insulin for 10 passages showed that a less than 2-fold increase in the gene expression of Nanog, Oct-4, GABRB3, GDF3, and DNMT3B were detected. Flow cytometry analysis of hESCs cultured with or without insulin for 10 passages showed that insulin increased the expression of stem cell markers Oct-4 from 27% to 64%, SSEA-4 from 67% to 100%, and TRA-1-81 from 39% to 75%.

5.4 The effect of oxygen tension on the self-renewal of human embryonic stem cells (III)

5.4.1 Cell proliferation and morphology under hypoxia

Hypoxic culture improved the hESC colony morphology in both RegES and hES culture media, resulting in more compact colonies with clearly defined borders and larger colony size. Under hypoxia in both culture conditions, only minor spontaneous differentiation was detected within cell colonies, whereas under normoxic conditions, spontaneous differentiation of hESCs at the center began within 5 days after passaging. An ELISA-based cell proliferation assay demonstrated that hypoxia increased the proliferation rate of hESCs maintained in the conventional hES culture medium by 42% ($p=0.001$) and in the RegES culture medium by 54% ($p<0.001$).

5.4.2 Hypoxia induces the expression of stem cell markers

No significant differences in the gene expression profiles of stem cell markers (Oct-4, Nanog, TDGF1, DNMTB3, GABRB3, and GDF3) were detected between hESCs cultured either in hES or RegES medium and maintained under hypoxic and normoxic conditions. To investigate the effect of O_2 tension on the translational expression of stem cell markers, flow cytometry analysis was performed on hESCs cultured under normoxic and hypoxic conditions. No significant differences were detected in the stem cell marker expression (Oct-4, SSEA-4) of hESCs cultured either in hES or RegES medium and maintained under hypoxia and normoxia at day 6. At day 8, however, flow cytometry analysis showed an increase in the expression of stem cell markers Oct-4 from 52% to 69% ($p<0.05$), SSEA-4 from 74% to 91%, and TRA-1-81 from 73% to 84% under hypoxia in both culture conditions.

5.4.3 Hypoxia alters the gene expression of several signalling pathways

To further investigate the possible molecular mechanisms activated by low O_2 tension, a customised quantitative real-time PCR array including genes from different signalling pathways was performed. Of the genes analysed, eight genes were upregulated and three genes were downregulated in hESCs cultured under hypoxic conditions in both culture media. In addition, 8 genes were upregulated in hESCs cultured under hypoxic conditions in the hES culture medium, whereas 2 genes were upregulated and 21 genes were downregulated in hESCs cultured in the RegES culture medium. The calcium and PKC pathway (CSF2, IL2, FOS, PRKCE, and TFRC) and several hypoxia signalling-related genes (DCTN2, PPARA, VEGFA) were altered under hypoxia in both culture conditions. The retinoic acid pathway was downregulated under hypoxia in hESCs cultured in the RegES culture medium.

5.5 Validation of the xeno-free medium formulation (IV)

5.5.1 Comparison of the RegES medium to HEScGRO (IV)

We compared the xeno-free RegES medium with the commercially available xeno-free HEScGRO medium specifically developed for hESC cultures using feeder cells. In the hESC line HS401, in contrast to RegES, the morphology of the hESC colonies revealed that the HEScGRO medium induced excess differentiation already during the adaptation process. After completion of the adaptation process, the HEScGRO medium was not able to maintain the undifferentiated growth of hESCs. The experiment was repeated two times.

5.5.2 Derivation, culture, and characterization of human embryonic stem cells (IV)

The optimised RegES medium was used to derive two hESC lines (Regea 07/046 and Regea 08/013). Both hESC lines exhibited characteristic hESC colony morphology, normal diploid karyotype, and were continuously cultured in RegES medium for over 80 passages. When stem cell marker expression levels were compared to the control hESC line Regea 06/040 derived and cultured in hES medium, both Regea 07/046 and Regea 08/013 cell lines exhibited higher expression levels of SSEA-4 (difference 8% and 2%, respectively) and TRA-1-81 (difference 10% and 11%, respectively). In qRT-PCR no significant differences (> 2-fold) in the expression levels of markers Nanog, Oct-4, GABRB3, GDF3, DNMT3B, and TDGF1 were detected between hESC lines derived in RegES and in control hESCs. Cell proliferation ELISA analysis showed that the cell proliferation rates of Regea 07/046 (A=0,286) and Regea 08/013 (A=0,272) were comparable to that of control cell line Regea 06/040 (A=0,287) cultured in hES medium.

5.5.3 Differentiation potential of human embryonic stem cells (IV)

The differentiation potential of the hESC lines derived and maintained in RegES medium was evaluated *in vitro* for EB-differentiation assay and *in vivo* for the teratoma formation analysis. The EB-derived cells from the hESC lines Regea 07/046 and 08/013 expressed markers from the three embryonic lineages; endoderm, ectoderm, and mesoderm, and structures from all these germ layers were also detected in the teratomas. In addition, we evaluated the *in vitro* differentiation potential of hESC line Regea 08/013 to cardiomyocytes and neural cell lineages. The cardiac and neural differentiation efficiency was similar in hES and RegES medium. Spontaneously beating cells that were positively stained with cardiac troponin T and vMHC markers exhibited a striated pattern. Neural cell differentiation was verified by the expression of neural precursor markers, neuronal markers and astrocytic markers in RT-PCR and the neuronal (MAP-2) and glial (GFAP) fate of the cells was confirmed with immunocytochemical staining.

5.5.4 Culture and characterization of induced pluripotent stem cells (IV)

In the present study, we cultured two iPS cell lines on hFFs in RegES medium. Both iPS cell lines were maintained in RegES medium for over 20 passages. The morphology of the cells was similar to that of the cells cultured in hES medium. In qRT-PCR no significant differences (over 2-fold) in the expression levels of stem cell markers Nanog, Oct-4, GABRB3, GDF3, DNMT3B, and TDGF1 were detected between iPS cell lines maintained in RegES and in hES medium. Only a 1% difference was detected in the flow cytometry analysis of both SSEA-4 and TRA-1-81 of iPS cell line 5-7 cultured in RegES and in hES medium, however, iPS cell line 6-14 maintained in hES culture medium exhibited higher expression of SSEA-4 (difference 17%) and TRA-1-81 (difference 21%) than iPS cells maintained in RegES medium.

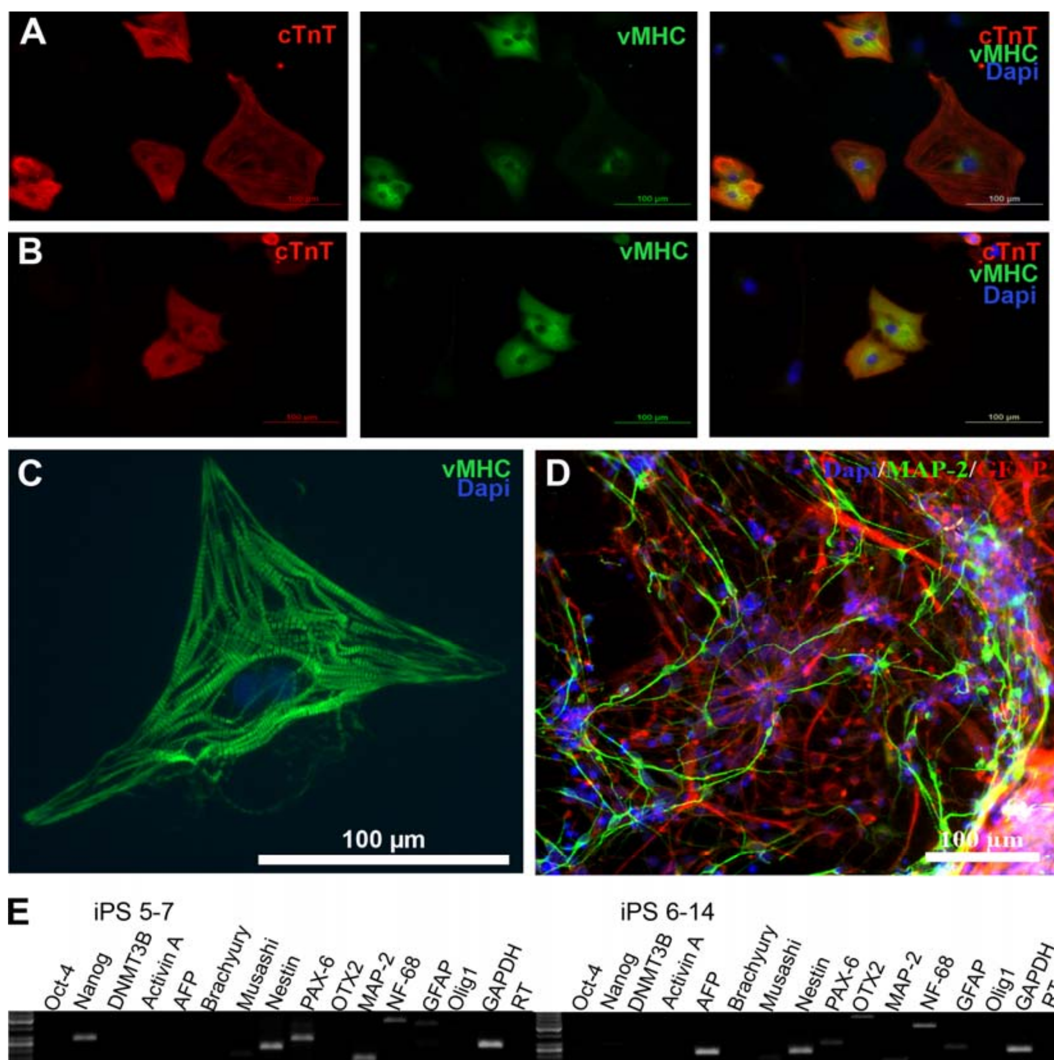

Figure 7. Analysis of pluripotency of the iPS cell lines maintained in xeno-free RegES medium. Differentiated cardiomyocytes from iPS lines 5-7 (A) and 6-14 (B) stained positive for cardiac troponin T (red) and vMHC (green), DAPI (blue). C) Higher magnification of vMHC staining. D) Most of the cells migrating out from the neurospheres stained positive for neuronal marker MAP-2 (green) and for astrocytic marker GFAP (red), DAPI (blue). E) RT-PCR analysis of neurospheres shows expression of neural precursor markers.

5.5.5 Differentiation potential of induced pluripotent stem cells (IV)

An *in vitro* EB differentiation assay demonstrated that both iPS cell lines cultured in RegES medium maintained their ability to differentiate into all three germ layers. The two iPS cell lines also differentiated *in vitro* into cardiomyocytes and neural cell lineages (Figure 7). Cardiomyocyte differentiation was confirmed based on the spontaneously beating cells exhibiting a striated pattern by immunocytochemical staining of cardiac troponin T and vMHC markers. Neural cell differentiation was verified by the expression of neural precursor markers, neuronal markers, and astrocytic marker in RT-PCR. No oligodendrocyte precursor marker Olig1 was detected in either cell line, while cell line 5-7 expressed Nanog and cell line 6-14 expressed endodermal marker α-fetoprotein after neuronal differentiation. The neuronal (MAP-2) and glial (GFAP) fate of the cells was confirmed in immunocytochemical staining.

5.5.6 Culture and characterization of adipose stem cells (IV)

ASCs isolated from adipose tissue samples were used to assess the performance of RegES medium for the culture of mesenchymal stem cells. AlloHS medium was used as a control in all experiments. Seven ASC lines were used for the WST-1 proliferation analysis performed at several time points (1, 4, 7, and 11 days). At day 4, hESC cultures with the RegES medium exhibited higher proliferation rates compared to the alloHS medium (p=0.035). Subsequently, ASCs continued to proliferate at a higher proliferation rate in RegES medium compared to alloHS medium at day 7 (p=0.022) and day 11 (p=0.018). Flow cytometric characterization of four cell lines was performed to compare surface marker expression characteristics of ASCs expanded in RegES and alloHS medium. The flow cytometry analysis revealed positive expression (>50% positive expression) for ASCs cultured in both culture conditions for the adhesion molecule CD105 and extracellular matrix protein CD90, while lacking the expression (<4%) of CD34 and CD45, markers of haematopoietic cell lineage. Additionally, no expression (<1%) of MHC Class II isotype HLA-DR was observed for ASCs cultured in alloHS or RegES media and only modest expression (10%) of MHC Class I isotype HLA-ABC was observed in ASCs expanded in RegES. While both culture conditions maintained the characteristic surface marker expression profile of ASCs, statistical analysis revealed significant differences in the expression of sialomucin-like adhesion molecule CD34 (p=0.043), leukocyte common antigen CD45 (p=0.017), adhesion molecule CD105 (p=0.020), and MHC Class I isotype HLA-ABC (p=0.021) of ASCs cultured in alloHS and RegES media.

5.5.7 Multipotential differentiation capacity of adipose stem cells (IV)

The multilineage differentiation potential of ASCs expanded in RegES medium was examined by culturing ASCs at passage 3 under conditions supporting osteogenic, adipogenic, and chondrogenic differentiation (Figure 8). The osteogenic induction was evident after 14 days of culture in osteogenic RegES medium and was

confirmed by ALP activity staining. Also, adipogenic differentiation was detected in osteogenic induction cultures with RegES. Already during the first week of adipogenic induction, accumulation of small lipid droplets was visible within the ASCs in adipogenic RegES medium. At day 14, ASCs in RegES adipogenic induction cultures proliferated rapidly and grew in a layered manner, with lipid droplets forming in several layers. Adipogenic differentiation was verified by oil red O staining as an indicator of intracellular lipid accumulation. In the chondrogenic induction cultures, ASCs expanded in chondrogenic RegES medium began to aggregate and form condensed pellets within 2 to 3 days after induction of chondrogenesis. The size of the condensing pellets grew rapidly, indicating profuse production of ECM. At day 14 cells stained with Alcian blue verified the presence of sulphated proteoglycans within the chondrogenic cell pellet.

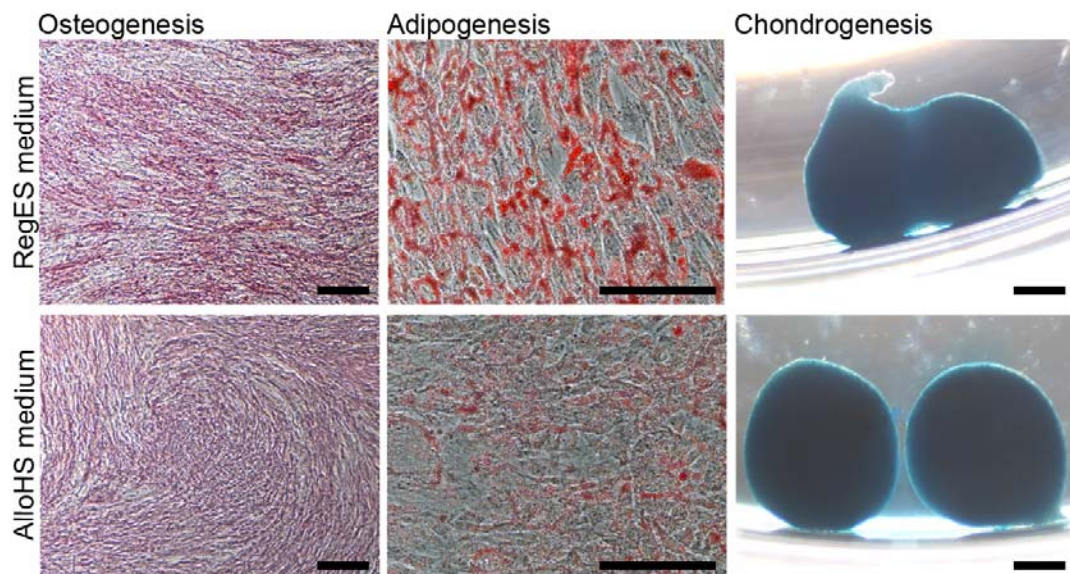

Figure 8. Multilineage differentiation potential of ASCs cultured in RegES and in control alloHS medium. ALP staining confirming osteogenic differentiation, oil red O staining confirming adipogenic differentiation, and Alcian blue staining confirming chondrogenic differentiation of ASCs. Scale bar 200 µm (osteogenesis and chondrogenesis) or 100 µm (adipogenesis).

6. Discussion

6.1 Automated culture, monitoring and analysis (I)

Generally, monitoring cell cultures requires time-consuming microscopy, manual imaging and image processing without automated data analysis. In the present study, an automated culturing, imaging and analysis system, Cell-IQ®, was used to develop an hESC colony area analysis method aimed at studying the growth dynamics of hESCs and enabling the comparison of different culture conditions. Traditionally, the growth rates of hESC colonies are manually counted from a single cell suspensions as doubling time during the exponential growth phase (Choo et al., 2006) or as an expansion of the area of single colony from photomicrographs (Koivisto et al., 2004). The automated culturing, imaging, and analysis system utilizing machine vision technology enables automatic observation, recording, and analysis of intact living cells. To validate the automated hESC colony area analysis method, the results of the automated analysis were validated using flow cytometry and immunocytochemical staining analysis. Based on this study, the automated monitoring system allowed for the reliable area measurement of live, intact cell colonies. There were no differences in the growth rates, amount of spontaneous differentiation, or in areas of undifferentiated and differentiated cells between the hESC colonies cultured in a standard incubator or in the automated system.

Using the developed analysis method, we examined the growth dynamics of two hESC lines. No differences in the average colony size and the growth rates between cell lines HS237 and HS293 were detected. It is not yet known, however, whether the size of the mechanically passaged cell aggregate influences the size of the resulting colony. To minimise variations in the cell aggregate size, the same person performed the mechanical passaging throughout the experiment. More undifferentiated areas were detected in the colonies of the HS293 cell line than in the colonies of HS237 cell line. Human ESC lines exhibit natural variation in growth rates and in their tendency to differentiate spontaneously in culture (Carpenter et al., 2009). In addition to analyzing the growth dynamics of hESCs, the automated monitoring system enabled the observation of hESC behavior, revealing more information than would be possible to discover by conventional microscopy observation. For example, the hESC colonies attached to the feeder cells on days 1 to 2 after passaging, and outgrowth of the hESC colonies began on day 3. Typically, spontaneous differentiation of undifferentiated colonies started 6 to 8 days after passaging in the center of the colonies and at day 7, the hESC colonies had reached a size where they needed to be passaged. Moreover, events such as apoptosis, cell division, cellular movement, and the number of single cells can be continuously observed and recorded in this system.

The automated culturing, imaging, and analysis system was also used to evaluate two different culture media for the culture of hESCs, the conventional hES culture medium and an X-vivo 10-based xeno-free culture medium. The results showed that in both HS237 and HS293 cell lines the average colony size and the undifferentiated colony area was smaller in colonies cultured in the xeno-free X-Vivo 10 medium indicating that hESCs from both cell lines grew better in hES medium. Based on this study, the automated cell culturing, imaging, and analysis system provides a useful tool for evaluating hESC cultures, allowing for continuous comparison of the effects of different culture media or different growth factor concentrations on cell growth and behavior. The analysis software of the system is relatively slow, however, allowing for the comparison of only few parameters in each experiment. Automation of culturing, monitoring, and analysis is essential for fast and reliable optimization of culture methods and differentiation protocols as well as for the robust mass production of clinically relevant quantities of the specific cell types for clinical application of stem cells.

6.2 Evaluation of xeno-free media formulations (II)

In this study, nine commercially available or published xeno-free media and serum replacements were systematically evaluated and compared to the conventional hES medium in hESC culture. Although these products have theoretical promise as serum alternatives in hESC culture, our results indicate that all the xeno-free formulations, except TeSR1 and human serum medium, were unable to maintain the undifferentiated growth of hESCs on human feeder cells. More specifically, the cells began to differentiate during the adaptation to the xeno-free test media and upon completion of the adaptation process, excess differentiation of the hESCs was evident. The loss of pluripotent cells was confirmed by the expression of SSEA-1 and loss of expression of Nanog. Moreover, colony growth rates decreased in the presence of X-Vivo 10, SSS, SerEx, and TeSR1. In addition, in the X-Vivo 10, X-Vivo 20, SR3, and Lipumin™ test conditions, the morphology of the hFFs was considerably altered, showing spherical appearance and premature detachment. Feeder cells are an important part of the culture niche, and therefore this might have had a critical effect on the excess differentiation observed among the hESCs.

The use of commercially available X-Vivo 10 medium was described previously by three research groups (Genbacev et al., 2005; Li et al., 2005; Peerani et al., 2007) for feeder cell-free culture of hESCs. Genbacev et al. used a high concentration of bFGF; Peerani et al. used TGFβ1 in addition to high bFGF concentration, and Li et al. supplemented X-Vivo 10 medium with a high bFGF concentration, stem cell factor (SCF), recombinant human flt3 ligand, and LIF. LIF, however, does not promote the self-renewal of hESCs (Thomson et al., 1998; Reubinoff et al., 2000; Humphrey et al., 2004). In addition, we used a low bFGF concentration (8 ng/ml) that is generally employed with feeder cell-dependent cultures and TGFβ1 in combination with X-Vivo 10 basal medium. It is unlikely that a higher bFGF concentration would have produced better results with these formulations, as a high bFGF concentration with feeder cell-dependent cultures generally results in a lower growth rate and colonies that grow upward instead of spreading, i.e., colonies that do not resemble the characteristic hESC colony morphology (K.R unpublished

observations). Besides the undesired effect on hFFs in some test conditions, it is impossible to evaluate what caused the excess differentiation of hESCs in the presence of these xeno-free formulations because as proprietary formulas, their constituents are unknown. It is probable that, rather than containing harmful components for hESCs, these formulations lack essential cues needed for the maintenance of hESC self-renewal.

Previously, Ludwig and co-workers reported feeder cell-free derivation and culture of hESCs using defined medium (TeSR1) including protein components solely from recombinant sources or purified from human material and ECM mix coating from human sources (Ludwig et al., 2006). They were able to derive two hESC lines in these xeno-free conditions, although neither one maintained a stable karyotype. Because the xeno-free formulation of TeSR1 was not commercially available when our study was conducted, we manufactured the medium according to the published report for our experiment. Based on our study, the TeSR1 medium was unable to maintain hESC self-renewal beyond 7 passages on human feeder cells. One obvious reason may be that TeSR1 was specifically developed to feeder cell-free culture of hESCs. Interestingly, albeit a low bFGF concentration was used, the hESC colonies cultured in TeSR1 medium did not have the characteristic hESC colony morphology or growth, but rather grew upward, resulting in plump colonies, which complicated the manual passaging. Based on EB-differentiation analysis an endoderm-specific marker was not detected, indicating defective pluripotency of hESCs cultured in TeSR1 medium.

In this study, we also evaluated medium containing human serum for the culture of hESCs. Previously, Richards and co-workers (Richards et al., 2002) derived an hESC line using 20% human serum medium and were able to propagate hESCs in an undifferentiated state for 10 passages. It was later observed, however, that prolonged use of human serum beyond the tenth passage led to the increased differentiation of hESCs (Richards et al., 2003). Based on the present study, the hESCs cultured in 10% and 20% serum medium underwent excessive differentiation during the initial adaptation phases and we were able to maintain hESCs in 10% human serum medium for only 9 passages. Similar results have been described before by Koivisto and co-workers who showed that hESCs underwent extensive spontaneous differentiation within a few passages in medium containing human serum (Koivisto et al., 2004). Interestingly, despite the excessive differentiation in the beginning of our experiment, the differentiation of hESCs decreased during subsequent passages, and although the hESC colonies were notably thinner in the presence of human serum than the hESCs cultured in hES medium, our results indicate that a population of cells was able to adapt to the culture conditions with human serum. More recently, however, Ellerström and co-workers derived and propagated an hESC line in 20% human serum medium for over 20 passages without excessive differentiation (Ellerström et al., 2006). The difference in the results may be due to different methods for preparing the serum, which yielded a different quality of the human serum used in the experiments, thus resulting in different effects on cell growth (Tallheden et al., 2005). The use of human serum in hESC culture medium is a xeno-free alternative to KO-SR, but like FBS, human serum is largely undefined and contains variable amounts of cytokines and growth factors, such as PDGF, epidermal growth factor (EGF) and BMPs that may influence hESC fate (Bieback et al., 2009). Human serum shows significant lot-to-

lot variability that may affect reproducibility, which means that each lot should be carefully tested prior to use (Caterson et al., 2002; Herrera and Inman, 2009). Commercial human sera are pooled from a heterogeneous group of donors. Pooling sera from selected donors might provide more homogenous sera for hESCs culture. In fact, Ellerström and co-workers used pooled blood from 15 donors and prepared the sera by using a previously described method (Tallheden et al., 2005; Ellerström et al., 2006), resulting in superior quality human serum. Nevertheless, yet another concern with the use of human serum is the potential transmission of serious pathogens like HIV, which has a long latent preclinical period and could go undetected in routine donor screenings (Mallon et al., 2006). By weighing the advantages and disadvantages, however, it can be concluded that the optimal approach for the xeno-problem of KO-SR would be the use of a defined serum replacement comprising purified human or recombinant components.

Control hES, TeSR1, and 20% human serum medium were used to evaluate the self-renewal of hESCs on feeder cell-free culture conditions using the previously described xeno-free ECM coating mixture (Ludwig et al., 2006). The attachment and colony formation in all these media, however, were poor. After the second passaging, only occasional colony formation was observed in hES and in 20% human serum medium, while no colony formation was observed in TeSR1 medium, indicating that none of these medium formulations was able to maintain self-renewal of hESCs in the absence of a feeder cell layer. Of note, other than the published report by Ludwig et al., no other reports describing the use of the xeno-free formulation of the TeSR1 medium have been published. Although published in 2006, only recently has the xeno-free TeSR1 medium formulation been made commercially available, indicating that it required several years of optimization before being ready for the market.

6.3 Optimization of xeno-free medium formulation (III, IV)

Due to the lack of commercially available xeno-free medium specifically developed for hESC culture, we developed a basic defined and xeno-free serum replacement formulation (RegES) comprising HSA, transferrin, amino acids, vitamins, antioxidants, trace minerals, and growth factors, including bFGF and insulin. Despite the fact that we were able to derive a new hESC line, Regea 06/015, using the xeno-free RegES medium and hFF cultured in human serum **(IV)**, the initial evaluations of the xeno-free RegES medium showed that the hESC colonies exhibited a thinner appearance and the growth rate of the colonies was slower than in the hESC lines cultured in the conventional hES medium, indicating that the formulation was not yet optimal for the culture of hESCs. Nevertheless, the first successful derivation of a new hESC line exhibiting a normal karyotype and hESC characteristics, including pluripotency in xeno-free conditions, demonstrated a vital proof-of-principle that our formulation was on the right track.

In this study, we identified essential nutrients and growth factors required for the undifferentiated growth of hESCs. To optimise the performance of the xeno-free RegES medium, we evaluated different basal media combined with RegES concentrate **(III)**. Based on this study, KO-DMEM had the best performance with

the RegES concentrate. Furthermore, from the results obtained, the following conclusion can be drawn. First, the undesired spherical morphology of the feeder cells observed with DMEM/F12 and in both DMEM formulations might have affected hESC self-renewal. Second, as the performance of DMEM high D-glucose was notably superior to DMEM low D-glucose, the high D-glucose concentration was beneficial to hESCs. Finally, DMEM/F12 also containing high D-glucose concentration might contain some detrimental component(s) for hESCs. Glucose is not just an energy source, but it also induces a variety of cellular signals. In fact, early human development *in vitro* is enhanced in medium lacking glucose (Conaghan et al., 1998). Therefore, as many of the differentiation protocols typically utilise media containing high glucose concentrations, such as DMEM/F12, the effect of glucose on the differentiation of hESCs into specific cell types should be further investigated. While we have demonstrated that glucose is important for the growth of hESCs, to our knowledge there are no previously published reports on the influence of glucose concentration on hESCs. High glucose levels increase the proliferation of mouse ESCs through the induction of peroxisome proliferator-activated receptor δ (PPARδ), which is at least partially mediated through the induction of cyclooxygenase-2 expression and PGE_2 (Kim and Han, 2008). Thus, the possible influence of the glucose on hESC self-renewal requires further investigation.

In addition to a high D-glucose concentration, the osmolarity of KO-DMEM was reduced to better mimic the natural environment of embryonic tissue. To further assess the optimal osmolarity, we evaluated an osmolarity range of 260-350 mOsm in the hESC culture for 5 passages. The optimal osmolarity for hESCs was approximately 320 mOsm, higher or lower osmolarity altered the morphology and restricted the growth of hESCs. In contrast to our results, Ludwig and coworkers previously reported that the optimal osmolarity for feeder cell-free hESCs culture is 350 mOsm, which in our experiment clearly restricted hESC growth (Ludwig et al., 2006).

Relatively few reports have been published on the effects of lipid-mediated signalling for the self-renewal of hESCs. Recently, Garcia-Gonzalo and co-workers demonstrated that the albumin-associated lipids present in KO-SR have a strong positive effect on hESC self-renewal (Garcia-Gonzalo and Izpisua Belmonte, 2008). Although they were able to indentify the major lipids bound to albumin and evaluated several lipid candidates, including lysophosphatidylcholine, LPA, S1P, PGE_2, and dexamethasone in hESC culture, the active lipid remains to be determined. We also observed a superior effect of AlbuMAX on hESC self-renewal and evaluated many different lipids and lipid derivatives in hESC culture. Based on our study, especially retinol, CLA and EPA improved the colony morphology and increased the number of undifferentiated colonies in both hES and RegES culture media **(III)**. The optimal performance of AlbuMAX may be due to synergism between the different lipids (Garcia-Gonzalo and Izpisua Belmonte, 2008), and therefore lipids with positive effects on hESC self-renewal should be further evaluated to determine optimal combinations and concentrations. Consistent with the data reported by Garcia-Gonzalo and co-workers (Garcia-Gonzalo and Izpisua Belmonte, 2008), in our experiment S1P neither stimulated self-renewal or proliferation nor prevented apoptosis, as described previously (Pebay et al., 2005; Inniss and Moore, 2006; Wong et al., 2007). In contrast, S1P induced excessive cell

death in both hES and RegES culture media. Although PGE_2 has been reported to be involved in the increase of proliferation of mouse ESCs in response to high glucose concentration (Kim and Han, 2008), in our experiment and consistent with previous results (Garcia-Gonzalo and Izpisua Belmonte (2008), PGE_2 resulted in poor morphology and excess differentiation in both culture media.

In the present study, we further evaluated the effects of retinol on hESC self-renewal. Retinol increased the proliferation of hESCs and induced the expression of the hESC-specific markers SSEA-4 and TRA-1-81. Consistent with the data obtained with mouse ESCs (Chen and Khillan, 2008; Chen et al., 2007), addition of retinol to the RegES formulation induced a high transcriptional expression of Nanog, a key transcription factor for self-renewal. Furthermore, based on this study, immunocytochemical analysis revealed increased expression of Nanog in the presence of retinol. Nevertheless, the increase in the translational expression of Nanog in response to retinol should be confirmed by flow cytometry analysis. In contrast to mouse ESCs, however, our results demonstrated that with hESCs the optimal effective concentration of retinol was ≥ 2.0 μM, while with mouse ESCs 0.5 μM retinol, an amount far less than the physiological concentration of retinol (1-2 μM), suppresses ESC differentiation (Chen et al., 2007; Goodman, 1984). While we detected no noteworthy difference between 2.0 μM and 3.5 μM retinol concentration, the retinol concentration of 0.5 μM did not increase the proliferation or the expression of Nanog to the extent detected with higher concentrations. Nevertheless, compared with published data with mouse ESCs (Chen et al., 2007; Chen and Khillan, 2008), our results indicate that retinol mediates hESC self-renewal via the overexpression of Nanog. Retinol plays an important role in a variety of essential biological functions, including vision, reproduction, growth, and development, and is typically associated with cell differentiation via its potent metabolite retinoic acid (Blomhoff and Blomhoff, 2006). The retinol-induced expression of Nanog in mouse ESCs is independent of retinoic acid, as well as of the JAK-Stat3, BMP2/4, and Wnt/β catenin signalling pathways (Chen et al., 2007; Chen and Khillan, 2008). Retinol was recently reported to regulate the self-renewal of mouse ESCs by activating PI3 kinase directly via IGF-II receptor/IRS-1, suggesting a growth factor like function of this molecule (Chen and Khillan, 2010). Whether this is also the mechanism of action in hESCs, however, remains to be elucidated.

In the present study, growth factor Activin A, which has long been known for its role in endodermal differentiation and cell growth (Sulzbacher et al., 2009), was also evaluated in the xeno-free RegES medium. Based on this experiment, Activin A stimulated the proliferation of the hESCs and increased the expression of the hESC-specific markers SSEA-4 and TRA-1-81. Previously, it has been shown that Activin A maintains the pluripotency of hESCs in the absence of feeder layers (Beattie et al., 2005; Vallier et al., 2005). Further, both mouse and human feeder cells secrete Activin A, although mouse feeder cells secrete higher levels (Eiselleova et al., 2008).

We also studied whether insulin is an important component of the xeno-free RegES medium formulation or whether it could be replaced with IGF-I, IGF-II or a combination of IGF-I, Activin A, and HRG-β1. IGF-II may act as a direct mediator of hESC growth, being expressed in response to bFGF and in parallel with

TGFβ/Nodal/Activin (Bendall et al., 2007). Basic FGF drives the expression of IGF-II and TGF-β factors from the hESC-derived fibroblast-like cells that generally surround the hESC colonies in both feeder and feeder cell-free cultures (Bendall et al., 2007). Based on our results, however, IGF-II could not replace insulin in the xeno-free RegES formulation. In addition, although Wang and co-workers suggested that insulin-initiated insulin receptor and IGF-I receptor signalling as well as low levels of bFGF are not sufficient to maintain hESC self-renewal (Wang et al., 2007), our results showed that insulin cannot be replaced with IGF-I alone, or a combination of Activin A, IGF-I, and HRG-β1, at least when the hESCs are cultured on a feeder cell layer. Human ESCs cultured without insulin and in the presence of the IGF-I, IGF-II, or a combination of Activin A, IGF-I, and HRG-β1, exhibited thin colonies with less clearly defined borders, as well as increased differentiation. Our results suggest that distinct signalling pathways mediate, at least in part, the effects of insulin and IGF-I or II in hESCs. Flow cytometry analysis of hESCs cultured with or without insulin showed that insulin increased the expression of the stem cell markers Oct-4, SSEA-4, and TRA-1-81, indicating that insulin was an important component of the xeno-free RegES formulation.

6.4 Hypoxia mediated self-renewal (III)

In the present study, we evaluated the effects of hypoxic conditions on the self-renewal of hESCs cultured in hES or RegES medium. Although the hypoxia system used in the present study was not optimal due to the need to manipulate the cells under normoxic conditions, exposure to hypoxic conditions prevented spontaneous differentiation, significantly increased proliferation, and supported hESC self-renewal, consistent with previous reports (Ezashi et al., 2005; Ludwig et al., 2006; Peura et al., 2007; Westfall et al., 2008; Forristal et al., 2009). The transcriptional expression of common genes associated with pluripotency was not significantly affected by hypoxia, which is consistent with previous reports (Forsyth et al., 2008; Westfall et al., 2008), however increased transcriptional expression of the stem cells markers Nanog, Oct-4 and Sox2 under hypoxia was recently reported (Forristal et al., 2009). The differences in the observed responses may be due to differences in hESC lines and culture conditions, including various degrees of hypoxia, exposure to normoxia during manipulation of the cells, culture media, feeder cells, and passaging methods. Furthermore, consistent with previous reports, there were no statistically significant differences detected in the transcriptional expression of hypoxia-inducible factors HIF-1α, HIF-2α, and HIF-3α under hypoxia (Forsyth et al., 2008; Westfall et al., 2008; Forristal et al., 2009). Investigation of the transcriptional expression of genes relating to different signalling pathways revealed that several hypoxia signalling-related genes and the calcium and PKC pathway were activated, probably in relation to the altered carbohydrate metabolism under hypoxic conditions. The retinoic acid pathway, generally associated with hESC differentiation (Inanç et al., 2008), was downregulated under hypoxia in hESCs cultured in only the RegES culture medium. Further analyses of the genetic mechanisms controlling self-renewal under hypoxia and verification of the results at the translational level are needed.

Although no statistically significant differences were detected in the translational expression of SSEA-4 and TRA-1-60, our results showed a consistent increase in the expression of these markers under hypoxia in all of the hESC lines. Consistent with previous reports, however, Oct-4 protein expression was significantly increased under hypoxic conditions, indicating a possible mechanism for hypoxia-induced self-renewal and the prevention of spontaneous differentiation (Covello et al., 2006; Forristal et al., 2009). Forristal and colleagues also proposed a model for HIF-mediated regulation of hypoxia-induced effects where HIF-3α regulates the expression of both HIF-1α and HIF-2α, and suggested that HIF-2α regulates hESC pluripotency and proliferation under hypoxic conditions (Figure 9).

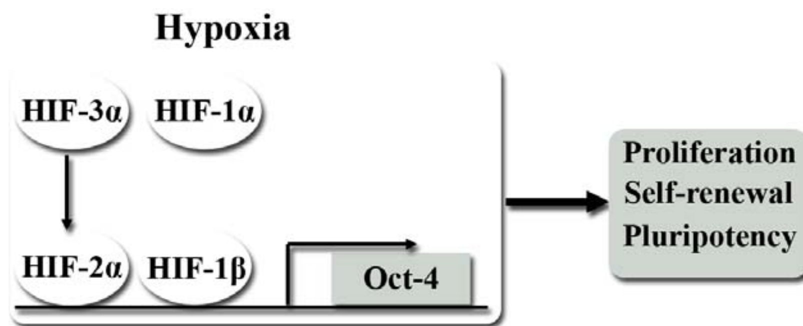

Figure 9. A proposed model depicting hypoxia induced transcriptional activity. In cells in which the Oct-4 locus is accessible as a result of open chromatin, its transcription is induced directly by HIF-2α/HIF-1β dimers in response to hypoxia.

6.5 Validation of xeno-free medium fromulation (IV)

In the present study **(IV),** the optimised xeno-free RegES medium was validated for use with hESC, iPS cell, and ASC cultures. Furthermore, RegES medium was compared to the first commercially available xeno-free medium, HEScGRO, specifically developed for hESC cultures on feeder cells. In contrast to a previous report in which hESCs were successfully maintained in HEScGRO medium for 10 passages on human feeder cells (Chin et al., 2009), we found HEScGRO medium to be inferior to RegES medium in terms of the amount of spontaneous hESC differentiation. HEScGRO is proprietary; therefore, it is not possible to evaluate the cause of the excess differentiation of hESCs in our experiment. HEScGRO contains 20 ng/ml bFGF, which is a much higher concentration than that in RegES medium (8 ng/ml). Feeder-cell free culture of hESCs using matrix from xeno-free hFFs together with HEScGRO supplemented with 100 ng/ml bFGF and ROCK inhibitor have also been described (Meng et al., 2008).

To date only two successful derivations of new hESC lines under xeno-free conditions have been described either by using XF-hFFs and human serum (Ellerström et al., 2006), or xeno-free ECM matrix and TeSR1 medium (Ludwig et al., 2006). The two hESC lines derived by using xeno-free ECM matrix and TeSR1 medium were karyotypically unstable (Ludwig et al., 2006). In study **IV**, we successfully derived new hESC lines (Regea 07/046 and Regea 08/013) in optimised

RegES medium on human feeder cells. Both cell lines were karyotypically normal. Furthermore, we continuously maintained these cell lines for over 80 passages in xeno-free conditions, which is far more than previously described (Ludwig et al., 2006; Ellerström et al., 2006). When compared to the hESC lines initially derived and maintained in conventional hES medium, these cell lines cultured in hES and RegES exhibited almost equal proliferation rates and transcriptional expression of stem cell markers. Although no statistically significant differences in the translational expression of stem cell markers were detected between these cell lines, both cell lines derived and maintained in RegES medium exhibited a modestly higher expression of SSEA-4 and TRA-1-81 compared to the hESC line cultured in hES medium.

When new xeno-free culture conditions are designed it is important to demonstrate that the differentiation potency is maintained. In the present study, we also demonstrated that structures from all three germ layers were detected when these hESC lines were differentiated *in vitro* and *in vivo*. Nevertheless, the direct differentiation of these hESC lines towards cardiomyocyte and neural cell lineages demonstrated that these cell lines efficiently differentiate to spontaneously beating cardiomyocytes and neural cells. Our results demonstrate that xeno- and serum-free derivation of hESCs is possible and these cells can be further differentiated to specific functional cell types.

Although the RegES medium was initially developed for the culture of hESCs, we further evaluated its performance in the culture of two iPS cell lines **(IV)**. The cell lines retained their characteristic morphology for more than 20 passages. Furthermore, no significant differences in the transcriptional expression of stem cells markers of the iPS cell lines maintained in RegES medium were detected compared to the control cell line maintained in hES medium. Moreover, no major differences in the translational expression of stem cell markers were detected between iPS cell line 5-7 maintained in RegES or in hES medium, while for unknown reasons iPS cell line 6-14 cultured in RegES exhibited lower expression of SSEA-4 and TRA-1-81 than iPS 6-14 cells maintained in hES medium. Both iPS cell lines generated structures from each germ layer when differentiated *in vitro* in EBs and differentiated to spontaneously beating cardiomyocytes and neural cells. Human somatic cells were recently reprogrammed to pluripotency using KO-SR XF or human plasma-derived cell culture additive and CellStart matrix (Rodriquez-Piza et al., 2010), or human serum and hDFs (Unger et al., 2009). Our results indicate that the xeno-free generation of human iPS cell lines is also possible by using RegES medium and further studies to address this question should be performed.

RegES medium was further evaluated for its ability to expand ASCs. The RegES medium supported significantly higher proliferation rates than ASCs cultured in alloHS medium, which is consistent with previous reports on ASCs expanded in xeno-free formulations compared to human serum (Lindroos et al., 2009). Furthermore, ASCs maintained their characteristic surface marker expression profile under xeno-free culture conditions although there were significant differences in the surface marker expression of ASCs cultured in human serum and RegES media **(IV)**. Different culture conditions affect the cell surface marker expression of ASCs (Lindroos et al., 2009). Nevertheless, irrespective of culture condition, the surface marker expression profiles presented in study **IV** are consistent with previous results

for ASCs (Gimble and Guilak 2003; Gronthos et al., 2001; Strem et al., 2005; Zannettino et al., 2008), with the expression of markers verifying the mesenchymal origin of cells, and the absence of markers of the haematopoietic origin of cells.

Moreover, ASCs exhibited multipotentiality towards osteogenic, chondrogenic, and adipogenic lineages in RegES medium (Figure 10). As previously described with other xeno-free media (Lindroos et al., 2009), the osteogenic differentiation capacity of ASCs cultured in RegES medium is less intense than that in alloHS medium, with simultaneous adipogenic differentiation occurring in the osteogenic induction cultures. One reason for this may be the superior cell proliferation rates in RegES medium, leading to cultures becoming rapidly confluent, which may support spontaneous trans-differentiation (Schilling et al., 2007; Liu et al., 2007; Lindroos et al., 2009) or due to dexamethasone supplementation of the osteogenic medium (Oshina et al., 2007). Also consistent with a previous report on other xeno-free media (Lindroos et al., 2009), RegES medium supported adipogenic and chondrogenic differentiation of ASCs. The differentiation protocols require further development with xeno-free media for optimal differentiation results.

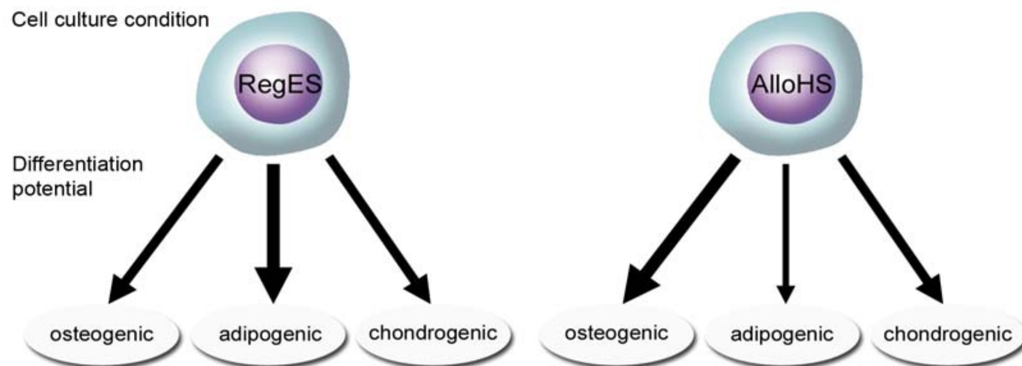

Figure 10. The differentiation potential of the ASCs in RegES and HS medium conditions. The thickness of the arrow indicates the differentiation potential towards each lineage. RegES=xeno-free culture medium; AlloHS= allogeneic human serum

6.6 Future perspectives

Since the discovery of stem cells, diverse culture conditions have been utilised for different types of stem cells. Most existing stem cell lines have been exposed to undefined xenogeneic products during *in vitro* establishment and expansion of the cells, which makes these cell lines undesirable for clinical applications. Furthermore, undefined products, such as FBS or human serum show significant batch-to-batch variability that may affect reproducibility of the results. At present, FBS or human serum is used for the *in vitro* expansion of adult stem cells. Various culture conditions have been described for the culture of hESCs, although the most widely used are KO-SR media employed with either human or mouse feeder cells and feeder cell-free culture utilizing Matrigel as a substrate and mTeSR1 medium.

Over the last few years, a number of molecular factors and signalling pathways that play a major role in maintaining self-renewal have been identified. Determining the

synergistic effects of these molecules and the convergence of independent signalling pathways affecting the growth and maintenance of stem cells will enable a better mechanistic understanding of stem cell regulation. Considerable progress has been made towards the generation of defined and xeno-free culture conditions for stem cells. An overview of the progress and anticipated future development in stem cell culture conditions are summarised in Figure 11. Currently, there are at least four commercially available xeno-free culture media for the culture of hESCs; HEScGRO (Millipore), developed for the culture of hESC lines with feeder cells; KnockOut SR Xeno-free (Invitrogen), which can be used with or without feeder cells; Stemedia™ NutriStem™ XF/FF (Stemgent) and TeSR2 (StemCell Technologies) for the culture of hESC lines in feeder-cell free conditions. Because human iPS cells have similar culture requirements with hESCs, the xeno-free culture media developed for hESCs will probably be applicable also for the culture of human iPS cells. A few xeno-free media have also been developed for the expansion of adult stem cells such as CellGro SCGM (CellGenix) and StemPro MSC FFM Xeno-Free (Invitrogen). In the present study, we also demonstrated that hESCs, iPS cells, and ASCs could be maintained in the same defined xeno-free medium formulation for a prolonged period of time while maintaining their characteristics. Furthermore, so far RegES is the only defined, xeno-free medium described that maintains the differentiation potential of stem cells both *in vitro* and *in vivo* after long-term culture.

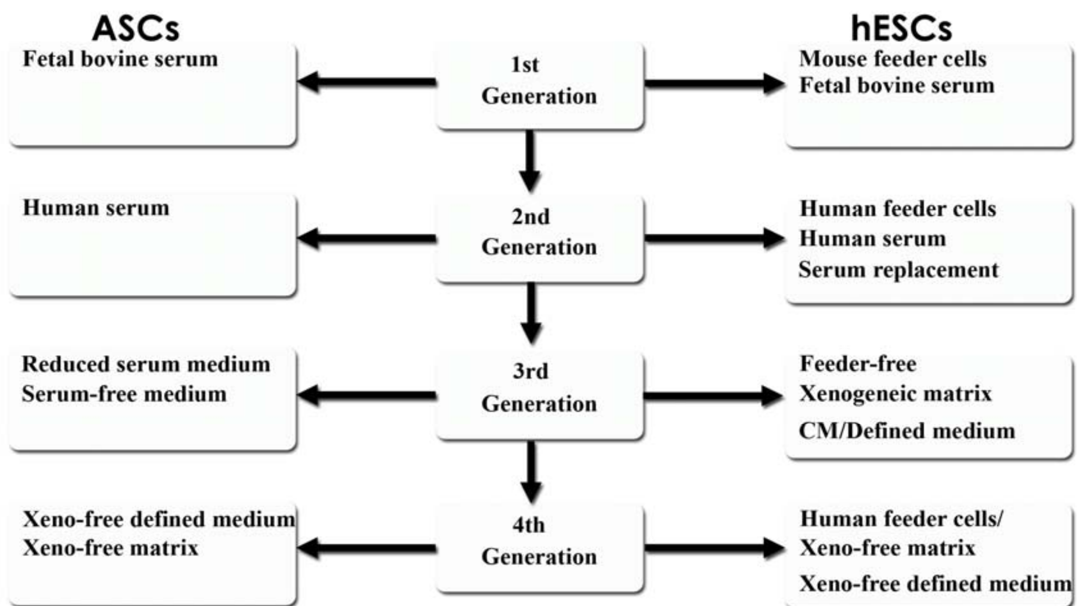

Figure 11. An overview of the history and future culture methods for ASCs and hESCs.

The new xeno-free products present an important advancement in the field of stem cell culture. Although all stem cell lines share the expression of characteristic stem cell markers, different culture conditions influence gene expression and therefore many of the cell properties (Skottman et al., 2006). The use of completely defined conditions will allow for a better understanding of stem cell regulations and provide more reproducible results. Nevertheless, it is essential to fully validate these products before they are brought to market. Future studies should focus on validating xeno-free culture conditions for long-term culture; maintaining the key

features of self-renewal, pluripotency, and genetic stability; as well as derivation, reprogramming, or isolation of new stem cell lines as a full proof-of-principle. These studies should also maximise proliferation rates, improve the efficiency of subcloning of these cells, and provide for scale-up to a manufacturing level. This is highly important not only for clinical use of these cells, but also for reliable research using these cells. Moreover, as many of the current differentiation protocols utilise a variety of undefined products, the xeno-free products should be tested as a base for differentiation protocols. The use of defined products in the differentiation protocols will facilitate the discovery of molecular mechanisms underlying the differentiation to various cell types and the reproducibility of differentiation processes.

Stem cells are invaluable tools for research and are a potential resource for regenerative therapies. As the number of potential applications for stem cell transplantation in the treatment of various degenerative diseases is rapidly increasing and several clinical trials using adult stem cells are ongoing as well as the first trial using hESCs is under way for acute spinal cord injuries, a strong focus on the safety and quality of stem cell transplants is needed. As the regulatory framework is constantly developing, we can only anticipate the future requirements for stem cell-based therapies. Although qualified xenogeneic reagents have previously received approval by the FDA, given the potential for infections and immune responses from xenogeneic products, it is preferable to maintain clinical-grade cells in defined, xeno-free conditions. By replacing xenogeneic products with defined xeno-free media formulation, the safety, reproducibility, and quality of the cells with therapeutic potential may be significantly enhanced. The basic RegES culture medium described in the present study has the potential to be further optimised for the xeno-free establishment, culture, and differentiation of various stem cell types and can ultimately serve as a platform for the production of clinical-grade stem cells and their derivatives for safer clinical cell therapy treatments. Additional pre-clinical safety and efficacy studies are also needed before the promise of the xeno-free products can be fully realised.

Although stem cells have the capacity to generate specific cell types and therefore may appear to be a panacea for cell and tissue replacement, there are still major challenges; including efficient differentiation of stem cells to fully functional cell types; changes in their epigenetic profiles, chromosomal aberrations during their establishment and maintenance; post-transplantation challenges like risk of tumors, genetic instability, and potential immunogenicity. It is clear that stem cells represent an equally large opportunity and challenge for the future of regenerative therapy. This new field of biology has been established with great enthusiasm, and huge amounts of work are being undertaken to dissect the basic biology and characteristics of these cells. Such understanding will undoubtedly uncover ways to harness their potential and bring the use of these unique cells into routine clinical practice.

7. Conclusions

The aim of this thesis was to develop and optimise defined and xeno-free culture conditions for stem cells to support feasible proliferation while maintaining the characteristics and differentiation potential of the cell types and meeting the regulatory standards for the clinical application of stem cells. Furthermore, these studies provided new insights into the molecular mechanisms that regulate the self-renewal and pluripotency of stem cells. The results of these studies led us to the following conclusions:

- The automated culturing, imaging, and analysis system Cell-IQ® enabled reliable analysis of the undifferentiated growth dynamics of hESCs, revealing more information than conventional microscopic observation. The system is applicable for the comparison of morphology and growth of hESCs in different culture conditions **(I)**.

- None of the xeno-free medium formulations evaluated in study **(II)** were able to maintain the self-renewal of hESCs on human feeder cells. A portion of hESCs was able to adapt to 20% human serum medium, but it was inferior to the conventional hES culture medium. Pooling sera from selected individuals and optimization of the serum preparation method may yield better quality human serum, decreasing the batch-to-batch variability, and increasing the reproducibility of the results. In the feeder cell-free culture of hESCs on a xeno-free matrix, the attachment and colony formation in 20% human serum medium, TeSR1, and hES medium were inadequate, indicating that none of these medium formulations was able to maintain hESC self-renewal in the absence of a feeder cell layer.

- Human ESC characteristics and proliferation were enhanced in the presence of KO-DMEM basal media, high glucose concentration, and osmolarity of 320 mOsm, together with xeno-free serum replacement RegES **(III)**. Specific lipids enhanced the growth and/or self-renewal of hESCs and could be used to supplement the xeno-free RegES medium.

- The addition of retinol in the RegES formulation **(III)** accelerated proliferation of hESCs and the expression of stem cell markers, especially Nanog, indicating that similar to mouse ESCs, retinol may mediate hESC self-renewal via Nanog overexpression. Activin A stimulated the proliferation of hESCs and increased the expression of stem cell markers. IGF-I, IGF-II or a combination of Activin A, IGF-I, and HRG-β1 could not effectively replace insulin in the xeno-free RegES culture medium. The lack of insulin in the xeno-free RegES medium resulted in a thin cell morphology and decreased the expression of stem cell markers.

- Exposure to hypoxic conditions prevented spontaneous differentiation, supported self-renewal, and significantly increased the proliferation of hESCs **(III)**. Although no major differences were detected in the expression of common genes associated with pluripotency or the hypoxia-inducible factors HIF-1α, HIF-2α, and HIF-3α, several hypoxia signalling-related genes and the calcium and PKC pathway were activated in both hES and RegES culture conditions. The retinoic acid pathway was downregulated by hypoxia in hESCs cultured in the RegES culture medium. Translational expression did not differ significantly, but the expression of SSEA-4 and TRA-1-60 consistently increased under hypoxia in all hESC lines. Oct-4 expression was significantly increased under hypoxic conditions, indicating a possible mechanism for hypoxia-induced self-renewal and the prevention of spontaneous differentiation.

- Xeno-free RegES medium enabled the derivation of new hESC lines on human feeder cells **(IV)**. These hESC lines were karyotypically stable over very long-term (> 80 passages) culture, and exhibited a feasible proliferation rate, characteristic stem cell marker expression, and pluripotency comparable to that of control hESC line.

- Xeno-free RegES medium enabled long-term (> 20 passages) culture of human iPS cells on a human feeder layer **(IV)** while maintaining characteristic stem cell marker expression. The iPS cell line 6-14 showed a lower expression of stem cell markers SSEA-4 and TRA-1-81 than iPS cells maintained in control hES medium. Nevertheless, pluripotency was maintained in both iPS cell lines cultured in RegES medium.

- The xeno-free RegES medium induced significantly higher proliferation rates of ASCs than alloHS medium, and maintained the multipotentiality towards osteogenic, chondrogenic, and adipogenic lineages, as well as the characteristic surface marker expression profile of ASCs. There were, however, significant differences in the surface marker expression of the ASCs cultured in alloHS and RegES media, **(IV)**.

- The results indicate that the basic xeno-free RegES medium is applicable for further optimization of xeno-free establishment, culture, and differentiation of various stem cell types and can ultimately serve as a platform for the production of clinical-grade multi- and pluripotent stem cells and their derivatives for safer clinical cell therapy treatments.

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