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Human pluripotent stem cells: glycomic approaches for culturing and characterization

Milla Mikkola
Program of Molecular Neurology,
Biomedicum Stem Cell Center,
University of Helsinki
Finland

ACADEMIC DISSERTATION
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Supervised by

Professor Timo Otonkoski, MD, PhD
Biomedicum Stem Cell Center, Program of Molecular Neurology,
University of Helsinki, Helsinki, Finland

and

Docent Timo Tuuri, PhD
Biomedicum Stem Cell Center,
University of Helsinki, Helsinki, Finland

Reviewed by

Professor Juha Partanen, PhD
Department of Biosciences, University of Helsinki, Helsinki, Finland

and

Professor Seppo Vainio, PhD
Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, University of Oulu, Oulu, Finland

Opponent

Professor Jari Koistinaho, MD, PhD
A.I. Virtanen Institute for Molecular Sciences, Laboratory of Molecular Brain Research, University of Eastern Finland, Kuopio, Finland

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Results! Why, man, I have gotten a lot of results. I know several thousand things that won't work.

Thomas A. Edison (1847-1931)

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by the Roman numerals I-IV.

I. Hovatta O, **Mikkola M**, Gertow K, Strömberg AM, Inzunza J, Hreinsson J, Rozell B, Blennow E, Andäng M, Ahrlund-Richter L: A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum Reprod. 2003 Jul;18(7):1404-9.*

II. **Mikkola M**, Olsson C, Palgi J, Ustinov J, Palomaki T, Horelli-Kuitunen N, Knuutila S, Lundin K, Otonkoski T, Tuuri T: Distinct differentiation characteristics of individual human embryonic stem cell lines. *BMC Dev Biol. 2006 Aug 8;6:40.*

III. Satomaa T*, Heiskanen A*, **Mikkola M**, Olsson C, Blomqvist M, Tiittanen M, Jaatinen T, Aitio O, Olonen A, Helin J, Hiltunen J, Natunen J, Tuuri T, Otonkoski T, Saarinen J, Laine J: The N-glycome of human embryonic stem cells. *BMC Cell Biol. 2009 Jun 2;10:42.*

IV. **Mikkola M**, Toivonen SC, Tamminen KM, Alfthan K, Tuuri T, Satomaa T, Natunen J, Natunen J, Saarinen J, Tiittanen M, Lampinen M, Valmu L, Partanen J, Otonkoski T: Lectin from *Erythrina cristagalli* supports undifferentiated growth and differentiation of human pluripotent stem cells**.** *Stem Cell Dev. 2012 October 29, (Epub).*

In addition, some unpublished data will be presented.

* equal contribution

ABBREVIATIONS

AFP	Alpha-fetoprotein
AP	Alkaline phosphatase
BMP	Bone morphogenetic protein
CM	Conditioned medium
DAPI	4,6-diamidino-2-phenylindole
DAZ	Deleted in azoospermia
DAZL	Deleted in azoospermia like
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	Dulbecco's modified Eagle's medium: nutrient mixture F-12
DMSO	Dimethyl sulfoxide
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ECA	*Erythrina Cristagalli* agglutinin
ECM	Extracellular matrix
EC	Embryonic carcinoma
EpiSC	Mouse epiblast stem cell
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	U.S Food and Drug Administration
FES	Finnish embryonic stem cell
FGF	Fibroblast growth factor
FiPS	Finnish induced pluripotent stem cell
FITC	Fluorescein isothiocyanate isomer 1
FOXA2	Forkhead box protein A2 (also known as HNF3β)
FUT	Fucosyltransferase
GABA	Gamma-aminobutyric acid
GCTM2	Germ cell tumor marker 2
hFF	Human foreskin fibroblast
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
hPSC	Human pluripotent stem cell
ICM	Inner cell mass
iPS	Induced pluripotent stem cell
IVF	In vitro fertilization
KLF4	Krüppel-like factor 4
KO-DMEM	Knockout™ -Dulbecco's modified Eagle's medium
KO-SR	Knockout serum replacement
$LacH_2O$	Lactose monohydrate
LacNac	N-acetyllactosamine
LnNT	Lacto-N-neotetraose
LIF	Leukaemia inhibitory factor
MAA	*Maackia amurensis* agglutinin
mEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cells
mRNA	Messenger ribonucleic acid
NEAA	Non-essential amino acids
NeuAc	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid

NF	Neurofilament
NMR	Nuclear magnetic resonance
N-glycan	Asparagine-linked glycan
OCT4	Octamer-4, POU class 5 homeobox 1
OncM	Oncostatin M
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGC	Primordial germ cell
PIWIL	Piwi-like 1
PSA	*Pisum sativum* agglutinin
PUM2	Pumilio homolog 2
PWA	*Phytolacca americana* agglutinin
qRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction
REX1	Zinc finger protein 42 homolog
ROCK	Rho-associated kinase inhibitor
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of the mean
SSEA	State-specific embryonic antigen
Sox2	Sex determing region Y- box 2
SRY	Sex determing region Y
STO	Murine embryonic fibroblast cell line
TEX14	Testis expressed 14
TGF-β	Transforming growth factor beta
Tra	Tumor related antigen
UEA I	*Ulex europaeus* agglutinin I
WFA	*Wisteria floribunda* agglutinin
XIST	X-inactive specific transcript

ABSTRACT

Human pluripotent stem cells (including embryonic stem cells and induced pluripotent stem cells) are defined by two important characteristics: unlimited self-renewal capacity and ability to switch on various differentiation pathways. These unique properties make them valuable tools for basic research and for the development of regenerative therapies. Since the discovery of stem cells, diverse culture conditions have been developed. Pluripotent stem cells are cultured either in the presence of feeder cells or with extracellular matrix components that together with growing colonies create a niche supporting the growth of undifferentiated cells. Current standard in vitro cell culture techniques are based on the use of xenogeneic reagents. However, this is not compatible with the clinical applications of human pluripotent stem cells.

The aim of this study was to develop optimal culture conditions for pluripotent stem cells without the use of xenogeneic reagents, based on the analysis of their cell surface glycan expression.

Initially, postnatal human feeder cells, foreskin fibroblasts, were shown to support the derivation of new embryonic stem cell lines and continued undifferentiated growth of these cells. However, the growth rate of stem cells was significantly lower on human feeder cells than on mouse embryonic fibroblasts. This feature restricts the use of human feeder cells for large-scale cell production of stem cells.

Next, a number of human embryonic stem cell lines were derived and characterized in detail. The results show that despite of similar basic characteristics in the undifferentiated state, the differentiation capacity of stem cell lines varies. This highlights the need to identify markers that reliably predict cell lineage propensity of the stem cells lines. To identify such predictive markers we conducted a global analysis of cell surface glycans expressed on pluripotent human stem cells. The results show that embryonic stem cells have a unique glycan fingerprint that differs from their differentiated derivatives. This suggests that information of stem cell surface glycans can be used to design markers that define specific stages of differentiation. In addition, this information can be used to develop defined reagents for stem cell culture.

Based on the analysis of stem cell surface glycans, specific glycan-binding lectins were studied for their capacity to support human pluripotent stem cells. This lead to

the discovery of a lectin, *Erythrina Cristagalli* (*ECA*) as a potent simple defined matrix for human pluripotent stem cells. This simple defined matrix, combined with a defined culture medium and the use of a Rho-kinase inhibitor at the time of cell propagation, allowed more efficient production of high-quality human pluripotent stem cells than Matrigel^{TM}, the current standard acellular matrix used for stem cell culture.

Taken together, these studies advance the development of technologies needed for the efficient generation of fully undifferentiated human pluripotent stem cells in defined conditions.

1. INTRODUCTION

Stem cells are present throughout life, from embryo to the adult. They are defined by their capacity for self-renewal and differentiation potential. Other cells in the body, do not have these abilities. According to their origin, stem cells are classified as embryonic, fetal, adult, or induced pluripotent stem cells. Fetal and adult stem cells, so called multipotent stem cells, are committed to specific developmental programs with limited self-renewal and differentiation capacity giving rise to only specific cell types. Stem cells in the fetus are needed for growth and maturation of the developing organs and tissues while the primary roles of adult stem cells in human are to repopulate the tissues by generating new mature cell types and regenerating damaged tissue in response to injury or disease (Choumerianou et al., 2008; Hipp and Atala, 2008; Pappa and Anagnou, 2009).

Pluripotency is the capacity of a cell to divide and renew itself but also to develop into different cell types in the organism. These two features make pluripotent stem cells invaluable tools for studying developmental biology and various diseases. The fertilised mammalian zygote enters the cleavage stage and divides several times and differentiates into outer trophoblast cells and the inner cell mass (ICM). Cell from ICM give rise to the pluripotent stem cells, embryonic stem (ES) cells. Current sources of human embryonic stem cells (hESCs) include excess embryos that would have been discarded after a successful in vitro fertilization process (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1995; Thomson et al. 1998). However, the use of IVF embryos has created debates about the ethics of stem cell research, and alternative sources for hESC have been studied. A breakthrough came few years ago, when researchers managed to reprogram somatic cells into pluripotent state by transduction with regulatory transcription factors (Takahashi and Yamanaka 2006; Takahashi et al 2007; Yu et al 2007). These cells are called induced pluripotent stem cells (iPS). However, the development of iPS methods has not replaced totally the use of hESCs but has offered additional insight into the biology of cell differentiation, dedifferentiation, and aging in new biological models.

2. REVIEW OF THE LITERATURE

2.1 PLURIPOTENT STEM CELLS

Pluripotency means the potential of a cell to develop into all different cell types found in an embryonic and adult organism, excluding extraembyonic organs, such as placenta and umbilical cord. Stem cells possess also enormous capacity to self-renew and due to these two unique properties pluripotent stem cells are attractive sources for cell-based therapies and regenerative medicine. Five different pluripotent stem cell types have been characterized, embryonic carcinoma cells, embryonic germ cells, embryonic stem cells, induced pluripotent stem cells and adult testis-derived germline stem cells, (Figure 1).

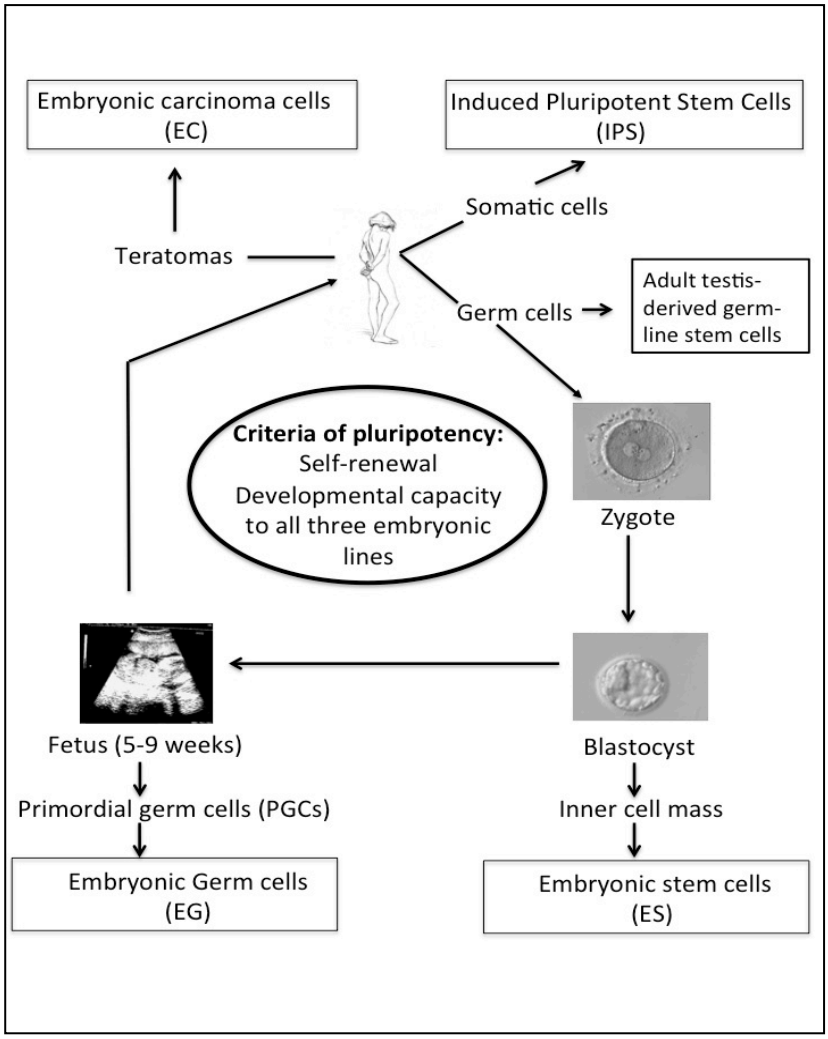

Figure 1. Origin of human pluripotent stem cells. Embryonic stem cells are derived from inner cell mass, embryonic germ cells from primordial germ cells and embryonic carcinoma cells are derived from teratomas. Induced pluripotent stem cells are reprogrammed from somatic cells and adult testis derived germ-line cells are dedifferentiated from spermatogonial stem cells. Figure modified from Eiges and Benvenisty 2002. Zygote and blastocyst images rights reserved VL-klinikat OY.

2.1.1 Embryonic carcinoma cells (EC cells)

Human pluripotent stem cells have also tumorigenic counterparts, embryonic carcinoma cells (ECs) (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1998), which are derived from gonadal tumors, teratocarcinomas. Teratocarcinoma consist of a mixture of differentiated cell types from all three embryonic germ layers (endoderm, mesoderm and ectoderm), as well as pluripotent cells, so called embryonic carcinoma cells (Martin and Evans 1975). ECs are usually aneuploid and often lack the ability to differentiate into well recognizable cell types even if some EC cell lines have been shown to differentiate quite well (Andrews et al. 1984; Pera et al. 1989; Andrews et al. 1990; Andrews et al. 1994). Numerous EC cell lines with variable differentiation capacity have been established. One of the best-characterized and most commonly used is NTERA2 (Andrews et al. 1984).

2.1.2 Embryonic germ cells (EG cells)

Human EG cells are isolated from primordial germ cells (PGCs) from embryonic gonads of five to nine weeks old fetuses (Shamblott et al. 1998). PGCs, the embryonic precursors of the gametes, undergo substantial epigenetic modifications resulting in erasure of imprints, genome wide demethylation, and in female foetuses X chromosomal reactivation. PGCs can be cultured at least for short periods as pluripotent stem cells (called embryonic germ cells (EG)), and they have been used to study human development. The importance of the EG cells as research material is currently moderately low.

2.1.3 Embryonic stem cells: from embryo to cell line

2.1.3.1 Mammalian preimplantation development

In normal mammalian development the presence of pluripotent stem cells is a transient phase during early embryonic development. Mammalian development is internal and the embryo receives nourishment from the mother. Embryogenesis starts after fertilization when the zygote has developed (Figure 2). Cleavage is a prolonged process from the early embryo to blastocyst implantation, and in comparison to non-mammalian organisms, the mammalian cleavage process is measured in days rather than hours. During the first 3-4 days the embryo divides roughly once a day When the embryo consists of approximately 16 cells, it is called a morula. The morula

continues to divide and the blastocyst cavity begins to form. The blastocyst consists of two cell types, small inner cell mass cells (ICM) (Figure 2) and outer superficial layer cells called trophectoderm. As the blastocyst develops, the cells in both cell types divide and the cavity enlarges. Subsequently, ICM begins to develop into a two-layered structure. The first layer consists of columnar cells called epiblasts and the second layer that lies closest to the blastocyst cavity consists of hypoblasts. During further development patterning of the embryo depends on signals provided by hypoblast to the epiblast (Coucouvanis and Martin 1995; Brook and Gardner 1997; Coucouvanis and Martin 1999).

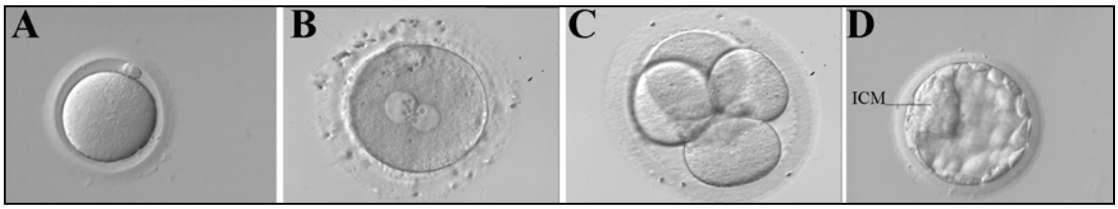

Figure 2. Embryo development from oocyte to blastocyst stage. A. Oocyte with one polar body. B. Fertilized oocyte, two pronuclei are seen in the middle. C. 4-cell stage embryo. D. Expanded blastocyst with clear and compact inner cell mass (ICM) and lots of cells in the trophectoderm. Image rights reserved VL-klinikat OY.

Epiblast cells have developmental potential to differentiate into all different cell types in the body. During gastrulation the epiblasts migrate and give rise to the three embryonic layers: endoderm (giving rise for example to lung, liver, pancreas and intestine), mesoderm (heart, blood, vasculature and skeletal muscle) and ectoderm (neurons, skin). After gastrulation these three layers will continue to proliferate into various organ-specific precursors during the embryonic period of prenatal development (Gilbert 1994; Aplin 1996; Carlson 1999; Murry and Keller 2008).

2.1.3.2 Derivation of Mouse ES and Epiblast stem cells

Embryonic stem cells (ES) are pluripotent stem cells most often derived from the inner cell mass of blastocysts. During the transition from ICM to embryonic stem cells, the normal development is changed – the cells acquire the capacity for unlimited self-renewal while maintaining the capacity to differentiate into all three germ layers (Tang et al. 2010).

The first mouse ES cell lines were derived from mouse blastocysts independently by two groups (Evans and Kaufman 1981; Martin 1981). The mouse ES cells expressed all the mouse EC cell markers and were capable of differentiation in vivo and in

vitro. The pattern of differentiation was essentially the same as for mouse EC cells.

Mouse epiblast stem cells (EpiSCs) are isolated from the early postimplantation mouse embryo just after implantation but prior to gastrulation (Brons et al. 2007; Tesar et al. 2007; Najm et al. 2011). Mouse ES and EpiSC are two distinct pluripotent states, representing cells of the preimplantation embryo and later epiblast cells.

2.1.3.3 Derivation of Human ES cells

In vitro fertilization (IVF) may provide a couple, who has been otherwise unable to conceive, a chance to establish a pregnancy. In IVF technology, the egg cells are fertilized by sperm outside the body. The embryos are grown in laboratory up to four-cell or blastocyst stage, and then placed in the uterus (Edwards 1981; Edwards et al. 1981). In vitro culture of cells from human blastocysts was first described in the early 1980s, and it was suggested that these cells might have potential application in regenerative therapies (Fishel et al. 1984; Edwards 2001; Edwards 2002).

Subsequent growth of human embryonic stem (ES)-like cells for two passages with partial characterization was first described by Bongso and colleagues in 1994 (Bongso et al. 1994), but the first permanent human embryonic stem cell (hESC) lines were reported in 1998 by Jamie Thomson's laboratory (Thomson et al. 1998). hESC lines have most commonly been derived from surplus blastocyst stage embryos created through IVF (Thomson et al. 1998; Reubinoff et al. 2000) but also from embryos at different developmental stages (Strelchenko et al. 2004; Zhang et al. 2006; Feki et al. 2008) and from single blastomeres of cleavage stage embryos (Klimanskaya et al. 2006; Eiges et al. 2007).

Although it seems that the hESC lines have a common genetic network of transcription factors that maintains them in a pluripotent state, there are reported differences between hESC lines. They may differ from each other in expression of several lineage markers (Abeyta et al. 2004; Rao et al. 2004; Ware et al. 2006; Adewumi et al. 2007). In female lines, variability has been shown in X-chromosome inactivation, inferred from X-inactive specific transcript (XIST) expression (Enver et al. 2005; Baker et al. 2007; Anguera et al. 2012). Reasons for differences between cell lines are still poorly understood. It has been discussed whether ES cells are trapped in development or if they are a result of culture conditions (Nichols et al.

2009). Recently a study (Tang et al. 2010) compared gene expression in single cells, following the development from the cells of the ICM through the outgrowth phase to the ES cell. Their results showed that the molecular signatures of undifferentiated ES cells are clearly different from cells of the ICM. Major changes were found in the expression of genes involved in the general metabolism, epigenetic regulators and also microRNAs. Human ES cells differ from mES but share defining features with the postimplantation epiblast state cells (EpiSCs), (Brons et al. 2007; Tesar et al. 2007; Silva et al. 2008; Vallier et al. 2009; Sun et al. 2012).

2.1.4 Induced pluripotent stem cells: from somatic cells to pluripotent cell line

Somatic cells cannot divide indefinitely and this is critical for maintaining the integrity of their derivatives. However, somatic cells can be reprogrammed into pluripotent state by nuclear transfer where the oocyte nucleus is replaced with a nucleus derived from a somatic cell, resulting in genetically identical ESCs to the donor (Wilmut et al. 1997; Wakayama et al. 2001). So far, there are no published reports of successful derivation of cloned hESC lines by nuclear transfer. Pluripotent cells have been generated from somatic cells also by cell fusion (Cowan et al 2005; Kim et al. 2007) but these cells have had an abnormal karyotype and imprinting status (Yamanaka 2008).

A breakthrough in reprogramming came when Takahashi and Yamanaka (Takahashi and Yamanaka 2006) selected 24 genes known to play a role in pluripotency, among them also genes known to be upregulated in tumors, and by using retroviruses introduced them into mouse fibroblasts. At the end of their studies they were able to produce ES-like cells from somatic cells by the ectopic expression of only four factors: Oct4, Sox2, Klf4 and c-Myc. These ESC-like cells showed similar results of pluripotency analysis than ES cells and they were named as induced pluripotent cells (iPS). First in the world, they (Takahashi et al. 2007) managed to reprogram human iPS cells from human dermal fibroblasts by using retroviral vectors, each carrying Oct4, Sox2, c-Myc, and Klf4, commonly called the Yamanaka factors. At the same time, another group (Yu et al. 2007) published very similar results of reprogramming. They used lentiviral vectors, each carrying Oct4, Sox2, Nanog and Lin28 to reprogram human fetal fibroblast to pluripotent stem cells.

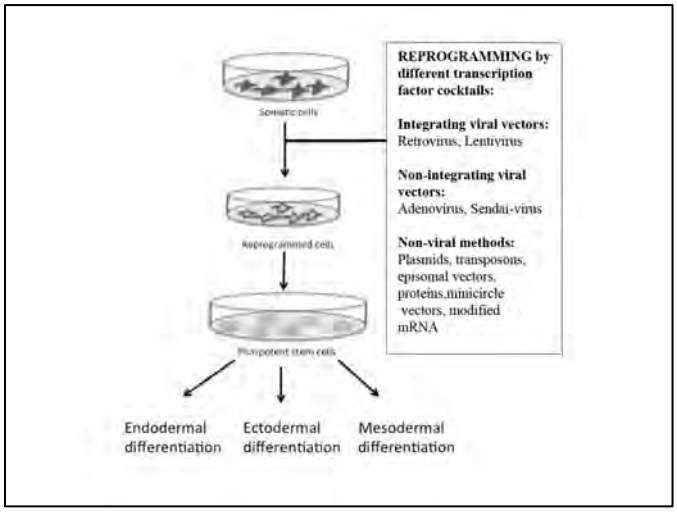

Figure 3. Induced pluripotent stem cell procedure. Somatic cells, like fibroblasts, keratinocytes etc., are reprogrammed with transcription factors. During a few days cells start to change their morphology and form stem cell like colonies. The formed colonies are picked, cultured and characterized, and the cells that fulfill the criteria of pluripotency can be used for further studies. Modified from Hussein and Nagy 2012 and http://rdnsystems.com.

Later, pluripotent stem cells have been induced from many different cell types by using different cocktails of the transcription factors Oct4, Sox2, Klf4, Lin-28, Nanog and c-Myc (Yu et al. 2007; Aasen et al. 2008; Park et al. 2008b; Giorgetti et al. 2009; Haase et al. 2009). Of these factors Oct4, Sox2, Nanog, and Lin-28 contribute to the reprogramming, and c-Myc and Klf4 enhance the efficiency of clonal recovery (Park et al. 2008a; Park et al. 2008b). Other factors have been identified that can replace the Yamanaka factors. Sox1 and Sox3 can replace Sox2, L-myc can replace c-myc, and Klf2 and Klf5 can replace Klf4 (Nakagawa et al. 2008).

Reprogramming is an inefficient and stochastic process with long latency. All somatic cells may have the potential to generate iPS cells but adult cells have proven to be more difficult to reprogram back to the pluripotent state than newborn or embryonic cells (Park et al. 2008b; Hanna et al. 2009, Liebau et al 2012). Human iPSC lines, generated independently in different laboratories, show very similar morphology and gene-expression profiles than hESC but also some differences exist (Maherali et al. 2007; Chin et al. 2009; de Souza 2010). The reprogramming process is associated with high mutation levels in early passages of newly established hiPSCs, but during culture the mutation load decreases through a selection process (Gore et al. 2011; Hussein et al. 2011). It has also indicated that early passage iPS cells still retain a partial epigenetic memory characteristic of the somatic cell of origin, and that such memory can influence the differentiation potential of these cells (Kim et al. 2009; Polo et al. 2010).

Retroviruses integrate into the cell's genome and may activate or inactivate critical host genes. This is a major safety concern for iPS cells in regenerative medicine. However, iPS technology development has moved rapidly since its discovery, and many alternative strategies to generate iPS cells minimizing or eliminating permanent modifications of the genome have been generated (Figure 3), such as adenoviral vectors (Stadtfeld et al. 2008; Zhou and Freed 2009), temperature-sensitive Sendai virus (Fusaki et al. 2009; Ban et al. 2011), transient transfection (Brambrink et al. 2008; Okita et al. 2008), single plasmids (Kaji, Norrby et al. 2009), removable transposon systems (Woltjen et al. 2009; Yusa et al. 2009), episomal vectors (Yu et al. 2009), and recombinant reprogramming proteins (Kim et al. 2009; Shao and Wu 2010). Moreover, the iPSCs can be established by the repeated administration of synthetic modified messenger RNA (Warren et al. 2010) and microRNA (miRNA) mediated reprogramming (Judson et al. 2009; Melton et al. 2010). At this point of rapid development, it is not obvious what the optimal reprogramming method will be but it appears likely that efficient pluripotent reprogramming is feasible without major genetic side effects.

2.1.5 Adult testis-derived germ-line stem cells

There have been reports of the derivation of pluripotent ES cell-like cells from the human testes (Conrad et al. 2008; Golestaneh et al. 2009; Kossack et al. 2009; Mizrak et al. 2010). In testes, the spermatogonial stem cells divide and differentiate to produce sperm but in pluripotent culture conditions *in vitro*, they can dedifferentiate to pluripotent ES-cell like cells. However, very recently, it has been shown (Chikhovskaya et al. 2012) that these cells share more features with multipotent human mesenchymal stem cells than pluripotent stem cells, but further research is required to assure the value of testis-derived germ-line stem cells.

2.2 CULTURING PLURIPOTENT HUMAN STEM CELLS

In vivo the pluripotent state is a transitory phase during early embryogenesis. The growth of pluripotent stem cells in *in vitro* conditions requires optimal balance between signals mediating cell survival, proliferation, and self-renewal. Most of the cells in the body need to attach to the extracellular matrix (ECM) in order to proliferate, migrate and differentiate, and even to survive. Human pluripotent stem cells (hPSCs) are not exceptions, they need either feeder-cells or extracellular matrix

coating, and, in addition, extrinsic signals from cell culture media to maintain pluripotency.

Pluripotent stem cells tend to maintain tight contacts with their neighbors and grow in colonies. They survive poorly when dissociated into single cells and hence exhibit poor cloning efficiency. This makes protocols for culturing and differentiation less robust, as colony size and spatial distribution of colonies can affect signalling pathways in ESCs (Peerani et al. 2009). For expansion of hPSC lines, it is crucial that cells maintain their undifferentiated state and their self-renewal capacity, and that they remain karyotypically normal. hPSCs are known to differentiate spontaneously if not kept under optimal culture conditions. Spontaneous differentiation can be induced by culturing hPSCs in suspension as aggregates, so called embryoid bodies (EBs), containing cell types from all three embryonic layers, endoderm, mesoderm and ectoderm (Thomson et al. 1995; Reubinoff et al. 2000; Odorico et al. 2001).

In contrast to other primary cultures, in optimal conditions hPSCs appear to be immortal and show no evidence of either crisis or senescence. However, several studies have reported that prolonged culture of cells in vitro may lead to the accumulation of genetic and epigenetic alterations, such as point mutations, gene copy number variation, chromosomal aberrations and aberrant DNA methylation, (Buzzard et al. 2004; Draper et al. 2004; Maitra et al. 2005; Carpenter et al. 2009; Narva et al. 2010; Hussein et al. 2011). These events may provide selective advantage to cells grown under prevailing conditions by reducing tendency for apoptosis and differentiation capacity. The genetic changes associated with culture adaptation often occur in cancer associated regions, (Reya et al. 2001; Herszfeld et al. 2006; Baker et al. 2007), and hence adapted cells could also give insights into mechanisms of cancer progression (Gokhale and Andrews 2012).

2.2.1 Feeder cell culture

Long-term hPSC cultures have generally been performed on a feeder cell layer, which functions to support the undifferentiated growth of hPSCs. However, using feeder cells is highly laborious and limits large-scale hPSC production. In addition, the use of feeders introduces undefined and poorly characterized parameters to the system, thus increasing variability.

2.2.1.1 Mouse feeder cells

On the basis of experience with mouse embryonic stem (mES) cells (Evans and Kaufman 1981; Martin 1981), the first human ES cell lines were cultured on mitotically inactivated mouse embryonic fibroblast (mEF) cells (Thomson et al. 1998). They are primary cells derived from day 12.5–13.5 fetuses, and they do not continue to proliferate indefinitely. mEFs are used optimally between passages 3 to 6, whereafter they begin to senesce and lose their capacity to support undifferentiated growth of hESC. Also immortalized mEFs (STO line) (Park et al. 2003) have been produced and used successfully as feeder cells.

The use of non-human materials bears a risk of transmitting pathogens, and they are not optimal in cultures aimed at cell transplantation in humans. For example, human stem cells cultured on mouse feeder cells or in conditions that contain components of animal-origin have been reported to contain a significant level of N-glycolylneuraminic (Neu5Gc) (Martin et al. 2005; Heiskanen et al. 2007). Humans have anti-Neu5Gc antibodies in the blood because this common mammalian sialic acid Neu5Gc is not synthesized in human cells. However, Neu5Gc expression in hPSC decreases upon culture in the absence of animal products (Heiskanen et al. 2007).

2.2.1.2 Human feeder cells

Efforts towards a xeno-free environment have resulted in the generation of several types of human feeder cells, including fetal and adult muscle and skin cells (Richards et al. 2002), adult fallopian tube epithelial cells, and human foreskin and placental fibroblast cells (Hovatta et al. 2003; Genbacev et al. 2005). Also bone marrow-derived stromal cells have been used as feeders (Cheng et al. 2003). In addition, autologous, spontaneously differentiated hESC-derived fibroblast-like cells can be used (Xu et al. 2004; Stojkovic et al. 2005; Wang et al. 2005).

The ability of different types of human feeder cells and cell lines derived from different mouse strains to support the undifferentiated growth of hESCs varies (Draper and Andrews 2002; Richards et al. 2003; Eiselleova et al. 2008). These variations in supporting the pluripotency and self-renewal of hPSCs can be attributed to the production of different types and amounts of secreted molecules. Comparing human and mouse feeder cells, human feeder cells (HFF) secrete Activin A and basic fibroblast growth factor (bFGF), which are known to be key factors in the

maintenance of the pluripotent state of stem cells, while mEFs secrete higher levels of Activin A but no bFGF at all. mEFs and HFFs secrete comparable levels of transforming growth factor beta 1 (TGFβ1), bone morphogenic protein 4 (BMP4) (Lim and Bodnar 2002; Prowse et al. 2005; Prowse et al. 2007; Eiselleova et al. 2008), and laminin-511 (Hongisto et al. 2012) that are thought to play a supportive role in the maintenance of hPSC survival and pluripotency (Miyazaki, et al. 2008; Evseenko et al. 2009; Vuoristo et al. 2009; Rodin et al. 2010)

2.2.2 Feeder-free culture

Maintenance of the undifferentiated stem cell phenotype in culture is not autonomous. It is dependent on the absence or inhibition of signals that stimulate differentiation. Together with extracellular matrix (ECM), the cell culture media should contain all necessary metabolites and nutrients to support either derivation or maintenance of hPSCs.

2.2.2.1 Extracellular matrix

The feeder layer provides important factors that suppress differentiation or promote self-renewal, and removal of the feeder cells will lead to differentiation. ECM consists of an organized, complex network of locally secreted macromolecules that govern many aspects of cellular behaviour, including proliferation and differentiation. *In vitro* growth matrices can be created from a variety of materials, both natural and artificial. Replacing feeder cells with defined human ECM components or synthetic biomaterials would be ideal for large-scale production of clinical-grade hESCs.

The first feeder-free hESC culture used Matrigel and laminin-coating and mEF-conditioned media. (Xu et al. 2001). Matrigel a soluble basement membrane extract from the Engelbreth-Holm-Swarm mouse tumor (Kleinman et al. 1982; Kleinman and Martin 2005), has been shown to support the culture of stem cells. It promotes the self-renewal and pluripotency of hPSCs. Matrigel contains mainly laminins, type IV collagen and enactin together with several growth factors such as bFGF, insulin-like growth factor-1, epidermal growth factor, platelet-derived growth factor (PDGF), nerve growth factor and transforming growth factor-b1, and proteins related to the binding and signaling of growth factors (Hughes et al. 2010). In 2005 hESCs were propagated first time directly onto Matrigel (Klimanskaya et al. 2005). The growth factor reduced (GFR) version of Matrigel has been modified to reduce the

levels of these growth factors (Vukicevic et al. 1992) and is widely used in stem cell culture. The main disadvantage of Matrigel is that its composition varies from batch to batch and it contains animal components, making it unacceptable for clinical grade cellular products.

It is difficult to elucidate the key interactions between cells and matrix because even single ECM proteins have multiple domains that can engage a variety of cell-surface receptors. Proteins such as laminins induce signals for promoting cell adhesion, growth, and migration. Laminins, the major components of the extracellular matrix of all basal laminae in vertebrates, are heterotrimeric glycoproteins forming 15 different combinations in human tissues (Tryggvason 1993; Aumailley et al. 2005). They bind collagen and proteoglycans, and integrate with cellular domains through integrins and other receptors. Many groups have studied specific laminins in stem cell culture (Miyazaki et al. 2008; Evseenko et al. 2009; Vuoristo et al. 2009; Rodin et al. 2010), and laminin-511 and laminin-521 have been shown to support growth of undifferentiated stem cells (Vuoristo et al. 2009; Rodin et al. 2010). Also other ECM proteins, such as fibronectin (Amit et al. 2004) and vitronectin (Braam et al. 2008), have been shown to support hESCs at least to some extent. These proteins carry the Arg-Gly-Asp (RGD) cell recognition sequence, which is recognized by integrins and mediates cell adhesion. However, none of these recombinant proteins are currently commonly used in routine stem cell culture, at least partly because of their high cost.

Synthetic materials could theoretically be an optimal solution as a stem cell matrix, because of reduced costs and elimination of possible hazardous contaminants. A synthetic chemical matrix based on the RGD sequence has been developed for hESC culture (Kolhar et al. 2010). Several commercial cell culture matrices are available. However, none of them has yet replaced Matrige^{TM} in common use.

The complex carbohydrates at cell surface play an essential role in the specificity of cell-cell interaction and cellular adhesion. Lectins, the carbohydrate-binding proteins, occur ubiquitously in nature and are abundant in all living organisms from bacteria to mammals. They serve many different biological functions from the regulation of cell adhesion to glycoprotein synthesis. They have been reported to enhance cell adhesion, growth and cell spreading (Grinnell and Feld 1979; Hughes et al. 1979; Rauvala et al. 1981; Rauvala and Hakomori 1981; Sanford and Harris-Hooker 1990; Blasco et al. 1995). The reported effects on cell proliferation have been specific for cell and lectin type. This suggests that specific interactions between lectins and cell surface glycans are key players in the potential growth support. In

hPSC research, lectins have been used for identification of pluripotent or differentiation stages (Wearne et al. 2006; Wearne et al. 2008; Toyoda et al. 2011; Wang et al. 2011), preferably based on exact data of stem cell glycans (Satomaa et al. 2009; Liang et al. 2010; Liang et al. 2011).

2.2.2.2 Soluble components

The cell culture media is used to provide necessary nutrients and metabolites for the maintenance of hPSCs. Both fetal bovine serum (FBS) and human serum (HS) have been used in culture media with feeder cells as well as in feeder-conditioned medium. The development of a serum alternative, the Knockout™ serum replacement (KO-SR), provided more standardized and defined culture conditions compared to serum-containing conditions. The mouse ES cell culture is depending on a lymphoid factor (Leucemia Inhibitory Factor, LIF), which promotes long-term maintenance of undifferentiated ESs by suppressing spontaneous differentiation (Smith et al. 1988; Williams et al. 1988). However, LIF is not essential to maintain the undifferentiated growth of hPSC or mEpiSC culture. Media containing KO-SR supports the prolonged growth of hPSCs in an undifferentiated state, with a higher growth rate and cloning efficiency than in serum-containing medium (Richards et al. 2002; Richards et al 2003; Amit et al. 2004; Koivisto et al. 2004). To understand the molecular mechanisms and signals, which maintain self-renewal of undifferentiated hPSCs, it is necessary to develop more defined culture systems. Over the last few years, a number of molecular factors and signalling pathways that play a major role in maintaining hPSC self-renewal have been identified (Table 1).

The FGFs are capable of eliciting a wide range of cellular responses such as proliferation, migration, differentiation, cell cycle arrest and self-renewal of hPSCs (Basilico and Moscatelli 1992; Hondermarck et al. 1994; Boilly et al. 2000; Kim, Cheon et al. 2005 Amit et al. 2004; Wang et al. 2005; Xu et al. 2005a; Xu et al. 2005b). FGF2, also known as bFGF, in particular is an essential component for the maintenance of hPSCs and withdrawal of bFGF (Kim et al. 2005) or inhibition of signalling through FGF receptors (Dvorak et al. 2005) results in hPSC differentiation. The exact molecular mechanisms linking FGF-signalling with pluripotency are actively studied. Recently, it was proposed that FGF signaling through ERK phosphorylation directly regulates OCT4 and associated proteins that establish and maintain pluripotency (Yu et al. 2011; Brumbaugh et al. 2012).

Many signals have been implicated in governing pluripotent stem cell fate together

with bFGF via direct and indirect mechanisms. The TGFβ growth factor family is of particular importance. Activin and nodal signalling activates the SMAD2/3 pathways (Xu et al. 2008; Vallier et al. 2009). SMAD2/3 bind to the promoter of the *NANOG* gene and activate its expression. Activation of SMAD2/3-mediated signalling or FGF2-mediated signalling also suppresses *BMP4* expression in human ES cells. In contrast, BMPs activate SMAD1/5/8 pathways, which inhibit *NANOG* expression in human ES cells (Xu et al. 2008) and induce differentiation (Greber et al. 2007). TGFβ-superfamily member GDF3, which is highly expressed in pluripotent stem cells in mice and humans (Pera et al. 2000; Skottman et al. 2005), blocks the BMP-mediated induction of differentiation and thus supports the maintenance of pluripotency marker expression in human ES cells (Chen et al. 2006).

Table 1. Basic factors in undifferentiated stem cell culture

Factor	**mES**	**mEpiSC**	**hPSC**	**hEC**
LIF	+	-	-	-
BMP4	+	-	-	-
Activin A and/or Nodal	-	+	+	-
FGF 2	-	+	+	-

+ the indicated growth factors are required for self renewal
- the indicated growth factors are not required for self renewal

Wnt signaling is known to play a role in pluripotency and self-renewal as well as in differentiation of both mouse and human ES cells. It is not required for self-renewal but canonical Wnt signalling is likely to promote the balance between self-renewal and differentiation in hPSCs. However, there is disagreement about the exact role of Wnt -mediated signalling in pluripotent stem cells (Sato et al. 2004; James et al. 2005; Sumi et al. 2008; Villa-Diaz et al. 2009; Blauwkamp et al. 2012; Davidson et al. 2012).

The first defined, completely animal-component free culture medium for hESC contained a combination of small molecules and proteins including bFGF, TGF-β1, litium chloride (LiCl), gamma-aminobutyric acid (GABA), and pipecolic acid. The cells were cultured on a matrix consisting of human collagen IV, vitronectin, laminin, and fibronectin (Ludwig et al. 2006). Several laboratories have designed completely defined culture conditions (Ludwig et al. 2006; Rajala et al. 2007; Akopian et al. 2010) and currently there are also many commercially available media. There is a lot of heterogeneity of culture conditions and also a lot of variety in the concentrations of growth factors in sustaining undifferentiated growth of hPSC. This reflects the fact that knowledge about the maintenance of self-renewal

and pluripotency of hPSC is still inadequate.

2.2.2.2.1 Stem cell fate compounds

Compounds that can bias the fate decision of stem cells have a long history in research, for example retinoic acid has been used to induce differentiation of both mouse and human embryonal carcinoma cells for over 30 years (Strickland and Mahdavi 1978; Andrews 1984). Small molecules, which can promote survival of hPSCs, have been successfully applied. Y-27632 (Watanabe et al. 2007) and pinacidil, an agonist of ATP-sensitive potassium channels, (Barbaric et al. 2010; Barbaric et al. 2011) inhibit Rho-kinase (ROCK) *in vitro.* ROCK is mainly involved in actin cytoskeleton rearrangement by promoting the stabilization of filamentous actin and triggers a signalling cascade that leads to coupling of actin–myosin filaments to the plasma membrane, resulting in actin–myosin contractility. Both pinacidil and Y-27632 promote survival of dissociated hPSCs by preventing apoptosis and increasing clonogenic capacity and hence can control human stem cell fates (Leung et al. 1996; Watanabe et al. 2007; Martin-Ibanez et al. 2008; Krawetz et al. 2009).

2.3 CHARACTERISTICS OF PLURIPOTENT STEM CELLS

hPSC cultures are complex mosaics of various cell types that cover the spectrum from self-renewing undifferentiated stem cells to incipient lineage-biased cells. The commitment of pluripotent cells to self-renewal or differentiation involves multiple genes and pathways and understanding the mechanisms controlling the status of hPSCs is a major challenge (Brivanlou and Darnell 2002).

2.3.1 Morphological characterization

Human pluripotent stem cell morphology is unique: they have a high nucleus to cytoplasm ratio, prominent nucleoli, and distinct colony morphology in epithelial cell-like colonies (Thomson et al. 1998; Reubinoff et al. 2000) (Figure 4). In addition, undifferentiated pluripotent cells have a distinctive nuclear architecture (lamina, nuclear speckles and heterochromatin domains) as well as chromatin structure (Meshorer and Misteli 2006). During differentiation, major changes in cellular morphology occur fast. After reprogramming many iPS colonies appear morphologically similar to ES cells but only a subset of these have comparable

molecular and functional features. Specific molecular and functional characterization is needed to distinguish fully reprogrammed iPS cells from those morphologically similar cells that are only partially reprogrammed and cannot form stable stem cell lines.

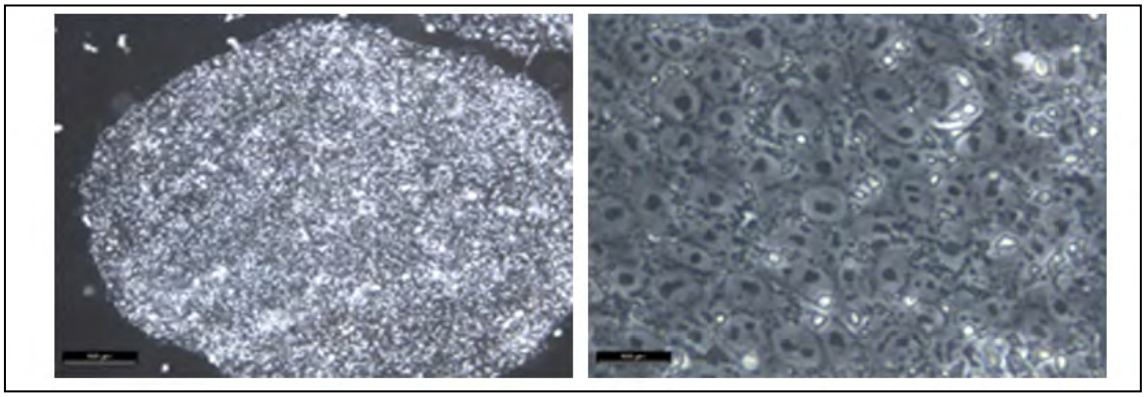

Figure 4. Undifferentiated morphology of pluripotent cells and colonies. Pluripotent cell colonies have sharp edges (A) and cells in the colonies are round with clear nucleoli (B). Scale bar 100 µm.

2.3.2 Cellular characterization

Crucial biological phenomena are mediated through carbohydrates that are displayed on the surface of each cell. The outmost layer of mammalian cells is covered with a dense layer of glycans, called the glycocalyx (Figure 5), which is comprised of glycoconjugates (glycoproteins, glycolipids, and proteoglycans). As glycosylation of individual cells changes during cell development and differentiation, the glycocalyx is characteristic to every individual cell type (Priatel et al. 2000; Moody et al. 2001; Comelli et al. 2006). The composition of the glycocalyx provides information used in cell recognition and in interactions with neighbouring cells and the environment (Varki A et al. 1999; Helenius and Aebi 2004).

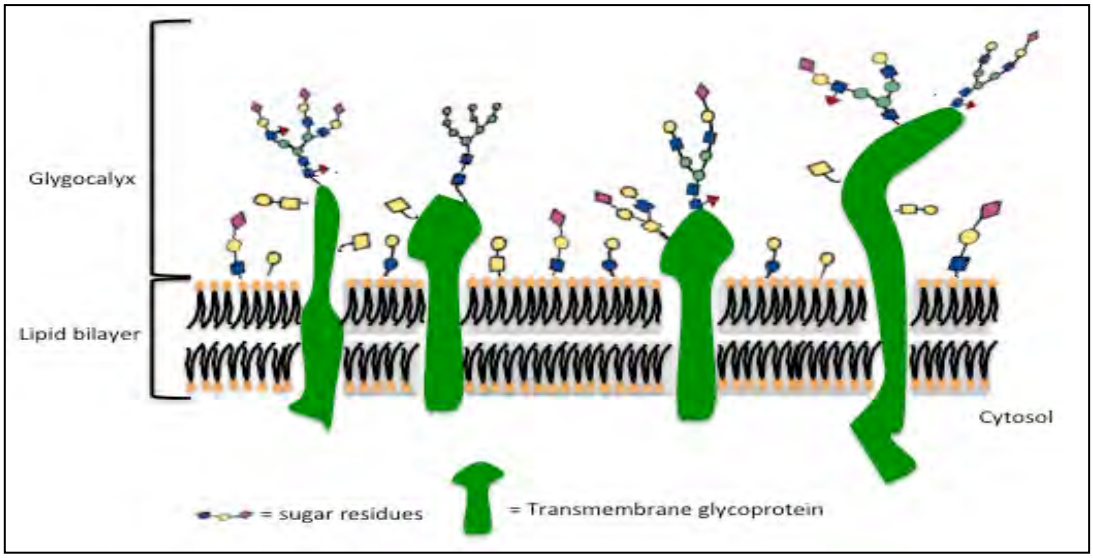

Figure 5. Schematic picture of glycogalyx. A dense layer of glycoconjugates cover all cells. Modified from Lanctot et al 2007.

2.3.3 Cell surface glycosylation

Glycans are considered to be some of the most structurally varied molecules in nature. Glycosylated structures on the cell surface have a role in cell adhesion, migration and proliferation (Duksin and Bornstein 1977; Sharon and Lis 1989; Arndt et al. 2011) and they vary considerably between species. Glycosylation is the most common posttranslational modification in proteins and lipids (Apweiler et al. 1999). The pattern of glycosylation cannot be predicted directly from gene expression because of the complex enzymatic machinery of glycosylation and also because of the variable availability and transportation of monosaccharides. Change in the activity of a single glycan biosynthetic enzyme may have drastic effects on the appearance of the cell, and a change in the expression of one glycan modifying gene may modulate multiple glycoproteins at once (Comelli et al. 2006).

There are two major types of glycosylation. In O-glycosylation the sugar is bound to the hydroxyl of a serine (Ser) or a threonine (Thr) residue. In N-glycosylation the sugar is attached to the amide group of an asparagine (Asn) residue in the consensus sequence Asn-X-Ser/Thr, where X represents any amino acid but proline (Petrescu et al. 2004). N-glycosylation affect many properties of glycoproteins (including their conformation, antigenicity, solubility, and recognition by glycan-binding proteins. Human N-glycans can be divided into biosynthetic groups of high-mannose-type, hybrid-type, and complex-N-glycans based on the amount and type pf glycan modification they have gone through (Figure 6) (Kornfeld and Kornfeld 1985; Schachter 1991).

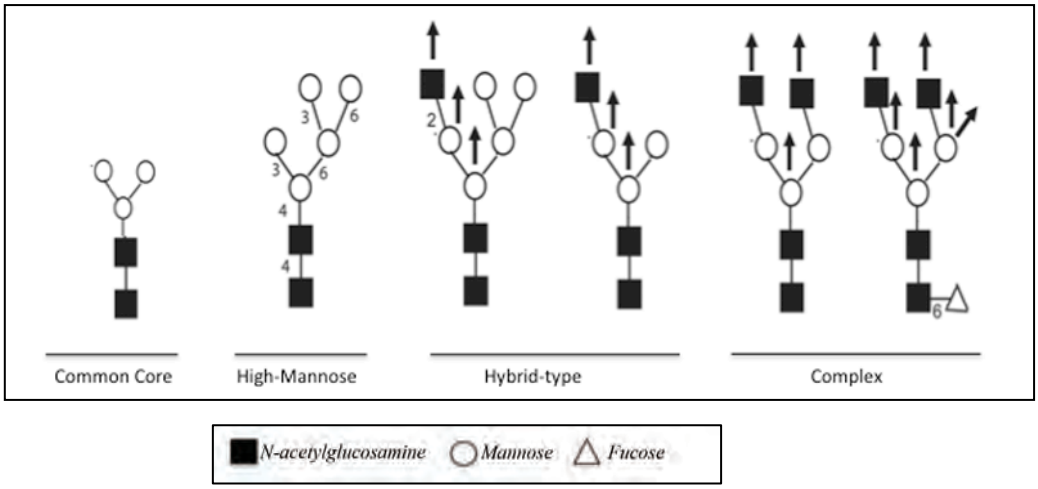

Figure 6. Overview of three N-glycan subtypes: high-mannose, hybrid and complex. Glycosidic linkages are indicated by lines connecting the monosaccharides, and arrows indicate the locations of branch formation in N-glycan diversification, not all of which occur in any single N-glycan. Modified from Essentials of glycobiology (Varki A, Cummings R et al. 1999).

Glycosylation takes place in the rough endoplasmic reticulum (RER), where a carbohydrate core is added to a newly synthesized protein. The protein is then transported from the ER to the Golgi apparatus (Yarema and Bertozzi 2001; Lowe and Marth 2003), where different intracellular, Golgi membrane-bound glycosyltransferases catalyse further reactions for growing oligosaccharide chains by using nucleotide sugar precursors as substrates (Baenziger 1994). Terminal epitopes, such as fucose residues or sialic acid, are added at the capping phase of the biosynthesis pathway by glycosyltransferases to bring more structural diversity to N-glycans. Fucosylation and sialylation are common types of glycosylation on the cell surface (Traving and Schauer 1998; Becker and Lowe 2003) and these modifications are critical also during embryogenesis and somatic stem cell differentiation (Landmesser et al. 1990; Shi and Stanley 2003; Xia et al. 2004). The glycosphingolipid composition changes during the differentiation of human embryonic stem cells and there are changes in the expression of specific pluripotency-associated glycosyltransferases (Chang et al. 2008; Satomaa et al. 2009; Liang et al. 2011; Wang et al. 2011). Fucosyltransferases, encoded by *FUT* genes, catalyse the transfer of fucose to terminal positions of glycolipid and glycoprotein carbohydrate groups. So far, nine human *FUT* genes that encode fucosyltransferases acting on N-glycans have been identified (Javaud et al. 2003). During hESC differentiation into EBs, the expression of *FUT1*, *FUT4* and *FUT8* disappears (Skottman et al. 2005; Satomaa et al. 2009; Hasehira et al. 2012)

Sialic acids are transferred to carbohydrate residues by sialyltransferases that

comprise a family of enzymes. During hESC differentiation into neural progenitor cells the expression of sialyltransferases *ST3GAL1*, *ST3GAL5* and *ST8SIA1* is up-regulated when compared to undifferentiated cells and, during definite endodermal differentiation, the expression of these sialyltransferases is down-regulated (Wang et al. 2011). These recent findings suggest that certain types of posttranslational glycosylation of cell surface proteins may reveal important information about transitions between pluripotent and differentiated states that occur during differentiation of pluripotent cells.

2.3.4 Cell surface antigens

Cell surface antigens have been long used to identify and purify specific cell types from blood and the immune system (Boyse et al. 1969) and since then they have played a key part in dissecting complex developmental systems. Human PSCs and ECs as well as mouse EpiSCs express a similar pattern of pluripotency associated cell surface antigens that are widely used to characterize the cells. Most of the commonly used cell surface antigens are glycoproteins or glycolipids (Kannagi et al. 1983a; Kannagi et al. 1983b; Andrews et al. 1984a; Andrews et al. 1984c). Regardless of the long history of these surface antigens, their biological functions are poorly understood.

Generally, a panel of surface antigen markers is used to monitor the differentiation status of hPSCs (Table 2) (Adewumi et al. 2007). Pluripotency associated cell surface markers that have been traditionally used to characterize hPSCs include tissue non-specific alkaline phosphatase markers Tra 2-49 and Tra 2-54, a high molecular weight antigen GCTM2, cell surface glycoprotein epitopes Tra-1-60 and Tra-1-81, glycosphingolipid antigen H type 1 and the stage-specific embryonic antigens SSEA-3 and SSEA-4.

The tissue non-specific alkaline phosphatase (AP) is expressed at high levels in all human pluripotent stem cells (hEC, hEG, hES and iPSC) and its expression levels decrease following stem cell differentiation. (Thomson et al. 1995; Shamblott et al. 1998; Takahashi et al. 2007). The expression of AP is usually monitored either by Tra 2-49 and Tra 2-54 antibodies or using enzymatic histochemistry.

Table 2. Most commonly used stem cell surface markers in pluripotent stem cell analysis.

Stem cell surface markers	mES	mEpiSC	hPSC	hEC
SSEA-1	+	+	-	-
SSEA-3	-	+	+	+
SSEA-4	-	+	+	+
Tra 1-60	+	+	+	+
Tra 1-81	+	+	+	+
GCMT 2		+	+	+
H-type 1	NE	NE	+	NE
Tra 2-49	+	+	+	+

+ the surface marker is expressed
- the surface marker is not expressed
NE not exminated

The high molecular weight antigen GCTM2 is associated with a cell surface keratan sulphate core molecule (Pera et al. 1988). Tra 1-60 and Tra 1-81 were earlier thought to recognize glycan epitopes of the same proteoglycan as GCTM2 (Badcock et al. 1999; Schopperle and DeWolf 2007) but recently it was shown that Tra-1-60 and Tra-1-81 may recognize type 1 lactosamine epitopes rather than keratan sulphate (Natunen et al. 2011). However, the exact molecular identity of Tra-1-60 and Tra-1-81 is still unknown.

Glycosphingolipids (GSLs) are biological building blocks that contain lipid and sugar residues with sphingosine attached on the cell membrane (Svennerholm 1980; van Echten and Sandhoff 1993). GSLs are key molecules required for many cellular processes like cell-cell and cell-matrix adhesion and differentiation (Fredman 1993; Varki 1993; Hemmoranta et al. 2007; Hakomori 2008; Satomaa et al. 2009a; Satomaa et al. 2009b). The presence or relative abundance of individual gangliosides may vary during the development but the major changes take place at the level of different GSL series. All GSLs are synthesized from a common precursor, lactosylceramide, via various biosynthetic pathways resulting in so-called lacto-series, globo-series and a-series ganglioside structures. All of these have been identified as pluripotency-associated GSLs (Figure 7.) (Satomaa et al. 2009b; Liang et al. 2010; Liang et al. 2011; Wang et al. 2011)

Lactoseries GSLs (Figure 7) were found in pluripotent stem cells quite recently (Liang et al. 2010; Liang et al. 2011). Of these the H antigens are fucosylated glycan units that are direct substrates for glycosyltransferases giving rise to A, B and Lewis blood group antigens. Lacto-series H-type I determinant (fucosyl Lc4Cer) is present in undifferentiated PSCs and it disappears during differentiation. This is apparently due to the reduction in fucosyltransferase FUT1/FUT2 expression (Liang

et al. 2010).

Of the globoseries glycolipids (Figure 7) SSEA-3 and SSEA-4 are among the most commonly used markers in identifying hPSCs. In mouse, SSEA-3 and SSEA-4 are expressed at cleavage stage embryos until morula stage but are absent from ICM and ES cells. In contrast, in human they are present in ICM and ES cells but are absent at earlier stages and their expression decreases rapidly upon differentiation (Andrews et al. 1982; Damjanov et al. 1982; Shevinsky et al. 1982; Thomson et al. 1998; Reubinoff et al. 2000). SSEA-3 is a precursor molecule to SSEA-4 and Globo-H. The sialylgalactosylgloboside SSEA-4 is a sialylated derivative of SSEA-3 and Globo-H (fucosylgalactosylgloboside) is a fucosylated derivative of SSEA-3. The most commonly used anti-SSEA-4 antibody (clone MC813-70) recognizes the latter globo-series structure but it also cross-reacts with a-series gangliosides GD1a and GM1b (Kannagi et al. 1983a; Kannagi et al. 1983b) that are shown to be present not only in undifferentiated cells but also in differentiated cells (Liang et al. 2010; Liang et al. 2011). This suggests that SSEA-4 (clone MC813-70), even if widely used, is not an optimal marker for undifferentiated stem cells.

It has been shown that SSEA3/4 negative stem cells have not lost their pluripotent differentiation capacity (Enver et al. 2005; Brimble et al. 2007) demonstrating that SSEA-3 and SSEA-4 are not essential for the maintenance of hPSC pluripotency. However, these antigens may play a role in embryonic development. SSEA-3 and SSEA-4 antigens belong to P-blood group system. Rare pp and p^k individuals lack P antigen (Gb4) and therefore also SSEA-3 and SSEA-4 antigens on their erythrocytes (Tippett et al. 1986). Women with this rare phenotype have a higher risk of spontaneous early abortion suggesting that SSEA-3 and SSEA-4 may play an important role in early development (Race RR 1975).

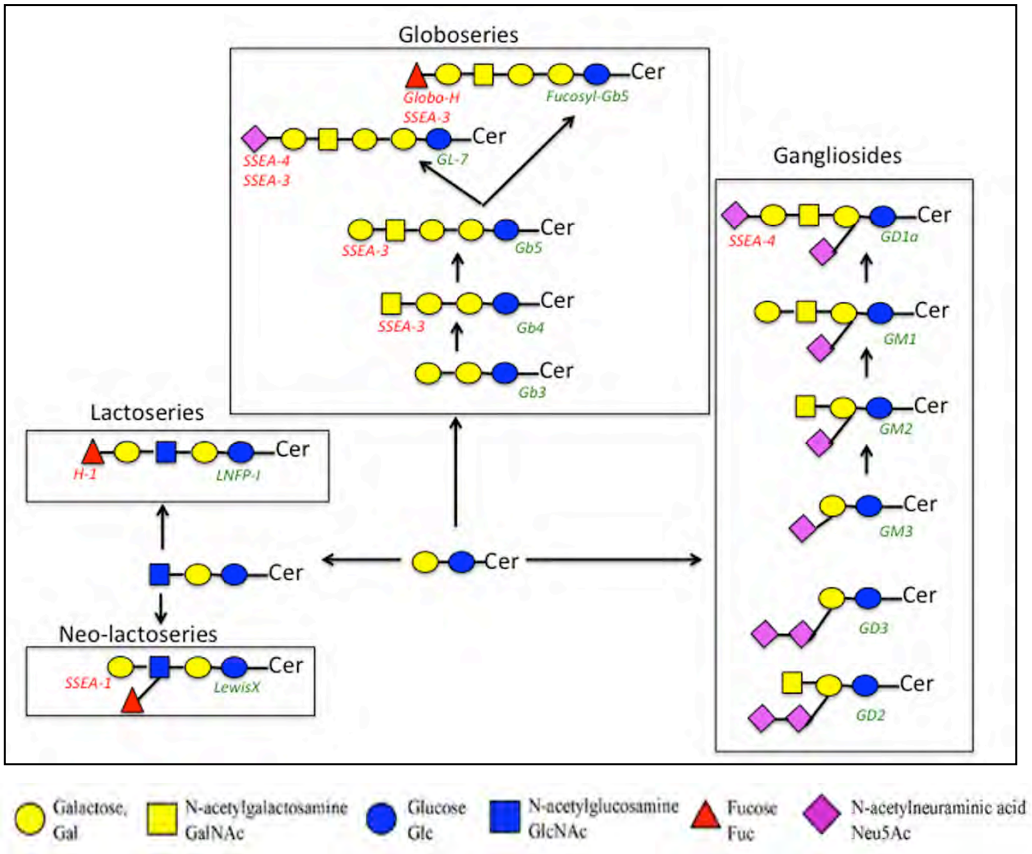

Figure 7. Illustration of the biosynthetic pathways leading to human stem cell specific GSL profiles. Globoseries and lactoseries GSLs are expressed on undifferentiated human ES cell membranes. Ganglioseries GSL are expressed mostly on differentiated hESCs even if some are found also on undifferentiated cells (Mikkola et al. unpublished data). The neo-lactoseries GSL, Lewis X, is expressed only on differentiated hESCs.

One important aspect in the characterization of stem cell quality is to assess the lack of markers for early differentiation. Undifferentiated hPSCs should not express the neo-lactoseries oligosaccharide antigen SSEA-1. SSEA-1 (also known as Lewis X and CD 15) is a terminal carbohydrate epitope, both on glycolipids and glycoproteins. This epitope is related to Lewis blood group antigens and is found in a variety of embryonic and adult tissues and cancers. As well as the globoseries glycolipid SSEA-3, also SSEA-1 expression pattern differs between human and mouse embryonic cells. In early embryonic mouse development, SSEA-1 is expressed from cleavage to blastocyst stage as well as in trophoblast but not in primitive endoderm (Solter and Knowles 1978; Gooi et al. 1981). In humans, SSEA-1 is observed in early embryonic development just at morula stage. Its expression increases upon differentiation in human cells, while in the mouse cells differentiation leads to decreased expression (Solter and Knowles 1978; Andrews et al. 1982; Shevinsky et al. 1982; Andrews et al. 1984a; Fenderson et al. 1987; Andrews et al. 1996; Thomson et al. 1998; Reubinoff et al. 2000). Fundamental

changes occur in GSL biosynthesis during hPSC differentiation. At first, cells begin to express SSEA-1 as well as ganglioseries GSLs while losing the expression of globoseries. This suggests that pluripotency-specific GSL groups are generally replaced by neolacto-series and ganglioside structures (Liang et al. 2010; Liang et al. 2011). Similar changes have also been described in hEC, teratocarcinoma (hTC) as well as mESC, indicating that it is a feature common to all embryonic cell types.

2.3.5 Genetic characterization

As genetic experiments cannot be carried out in the early human embryo, confirming the role of the key transcription factors in early embryonic development is impossible. Studies are limited to investigating the effect of their manipulation *in vitro*. Studies with induced pluripotent stem cells have increased our understanding of the mechanism of key transcription factors in early development.

The transcription factors Oct4 (also known as Pou5f), Sox2, a member of the SRY-related high mobility group -box (SOX) family and Nanog, a homeobox protein, are key regulators of early development. Oct4 is required to maintain the pluripotent cell population of the ICM and epiblast and without it, ICM and epiblast cells will differentiate along the trophoblastic lineage (Scholer et al. 1989; Rosner et al. 1990; Scholer et al. 1990a; Scholert et al. 1990b; Nichols et al. 1998). Sox2 is first found in the morula stage embryo and later becomes restricted to the ICM and epiblast and then to the germ cells. Sox2 is also expressed in the extraembryonic ectoderm and in the precursor cells of the developing central nervous system (Gubbay et al. 1990; Avilion et al. 2003). Nanog was identified as a pluripotency factor over ten years later than Oct4 and Sox2 (Chambers et al. 2003; Mitsui et al. 2003). Its expression is required at a later point than Oct4 but it is also restricted to the ICM and epiblast. Nanog is downregulated at the time of implantation, then re-expressed again in the posterior region of the post-implantation egg cylinder (Hart et al. 2004).

OCT4-SOX2-NANOG-triad is central to the transcriptional regulatory hierarchy that specifies the identity of hPSCs. The three factors promote pluripotency and self-renewal through interconnected autoregulatory and feed-forward loops (Figure 9). These transcription factors regulate hundreds of target genes in pluripotent cells, indicative of their pivotal role in pluripotency. They bind to transcriptionally active genes that have a role in pluripotency and transcriptionally inactive genes that promote lineage commitment. However, the expression of this triad does not, in

itself, guarantee pluripotency indicating that other factors are also needed (Scholer et al. 1990b; Nichols et al. 1998; Avilion et al. 2003; Chambers et al. 2003; Lee et al. 2004).

Kruppel-like factor 4 (Klf 4) is another member in the pluripotency core concert. Klfs play critical roles in many biological processes such as proliferation, differentiation, proliferation, and apoptosis (Garrett-Sinha et al. 1996; Shields et al. 1996; Rowland et al. 2005). Klf4 plays an essential role in the pluripotency and its overexpression inhibits differentiation of PSCs. (Li et al. 2005; Takahashi and Yamanaka 2006; Takahashi et al. 2007). It interacts directly with Oct4 and Sox2 and is required for transcriptional activation of Nanog. The highest levels of activation were observed when these factors were accompanied by Oct4 and Sox2, indicating that all four act synergistically to maintain self-renewal and pluripotency (Kim et al. 2008; Wei et al. 2009).

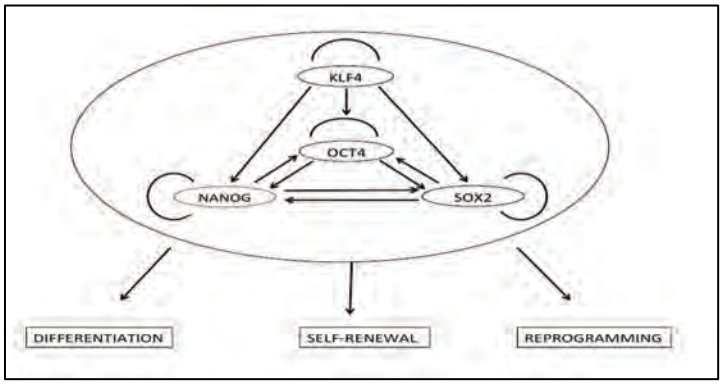

Figure 9. In hPSC cells, OCT4- SOX2-NANOG triad form a core transcriptional network. KLF4 is an upstream regulator of feed-forward transcription loops. Modified from Kim et al (Kim, Chu et al. 2008).

2.3.6 Genomic and Epigenetic characterization

Telomerase activity is essential for continued replication in any cell line. The telomerase enzyme is a ribonucleoprotein that maintains telomere length. A high level of telomerase activity is associated with cell proliferation during embryonic development but also with malignant growth. Telomerase is highly expressed in germ cells but somatic cells do not express it. Their telomeres shorten during senescence. This is related to the reduction of life span by limiting the number of cell division. hPSCs express high levels of telomerase, which explains their ability to undergo practically unlimited self-renewal (Andrews et al. 1986; Thomson et al. 1998; Reubinoff et al. 2000).

Somatic mutations are not an unusual phenomenon in dividing cells and they can generate a selective advantage, such as a greater propensity for self-renewal. Human ES cells as well as hiPSC were found to have a tendency to acquire changes on prolonged culture, commonly affecting chromosomes 12, 17, 20 and X or certain fragments of these chromosomes (Draper et al. 2004b; Hoffman and Carpenter 2005a,b; Mitalipova et al. 2005; Spits et al. 2008; Mayshar et al. 2010). Especially the changes in chromosome 12 and 17 that seem to provide growth advantage in prolonged culture are also common in testicular tumors (Sandberg et al. 1996; Looijenga et al. 2003). It has been suggested that the actual method of passaging, especially trypsinization, may affect the accumulation of cytogenetic abnormalities (Brimble et al. 2004; Mitalipova et al. 2005). Another explanation for this phenomenon could be the density of colonies. The high cell density within a colony may predispose cells to a greater metabolic stress, which may lead to the selection of the cells that are more resistant to stress (Buzzard et al. 2004).

The balance between self-renewal and differentiation is crucial for development and for the health of an individual. Epigenetics refers to the underlying mechanism of coordinated gene control that affects gene expression without changes in DNA sequence. Waddington (Waddington 1959; Waddington and Robertson 1966) developed a concept "canalization", which models the developmental status and epigenetic landscape. The epigenetic landscape of a cell is likely to be an indicator of its past and current developmental state and may predict its future potential. In other words, genes restrict the extent to which the environment can influence the phenotype. Epigenetic modifications including DNA methylation, histone modifications and the activities of small interfering RNAs are important for the expression of pluripotency-associated genes, thereby affecting pluripotency and differentiation (Jaenisch and Bird 2003; Hoffman and Carpenter 2005b; Richards 2006). Recent technological advances have led to comprehensive epigenome reference maps and data of epigenetic modifications in mouse and human pluripotent cells (Meissner et al. 2008; Laurent et al. 2010; Doege et al. 2012) have significantly increased our understanding of ES cells and epigenetic reprogramming. However, the epigenetic remodelling during human development and reprogramming is still in the beginning.

2.3.7 Demonstration of pluripotency

The differentiation capacity of human pluripotent cells can be studied both *in vitro* and *in vivo.* True pluripotent cells can be differentiated as embryoid bodies (EBs). EBs are compact spheres that contain loosely organized tissues that recapitulate the normal developmental processes of early embryonic stages and promote the cell–cell interaction required for cell differentiation. Through EB differentiation markers of each of the three germ layers can be assessed.

Germ line transmission in chimeric animals is the golden standard for demonstrating the pluripotency of mouse embryonic stem cells. For obvious ethical reasons, the ability of human pluripotent stem cells to contribute to the germ line is not possible. The teratoma assay is the current functional standard to demonstrate the pluripotency of human stem cells (Adewumi et al. 2007). Teratomas are tumours containing differentiated elements of all three embryonic germ layers. In this functional *in vivo* pluripotency assay, the spontaneous differentiation potential of human pluripotent stem cells is measured after their injection into immunodeficient mice (Adewumi et al. 2007; Gertow et al. 2007). If the cells are pluripotent, they will form well-differentiated tumours and sections of the tumor will show elements from all three germ layers (Damjanov and Solter 1974; Andrews 2002; Solter 2006).

2.4 SUMMARY OF PLURIPOTENCY

Pluripotency does not represent a single defined stage. In order to understand the distinct properties of pluripotent cells and molecular mechanisms behind these, global gene, glycan and protein expression profiles and epigenetic modification should be investigated. Table 3 summarizes the most common characteristics of pluripotent cells (mES, EpiSC, hPSC and hEC) grown *in vitro*. The properties of pluripotent stem cells from adult testes are controversial (Mizrak et al. 2010, Chikhovskaya et al. 2012) so these cells are not included in table 3.

Table 3. Properties of various pluripotent cell populations in vitro (modified from Pera and Tam 2010).

Response to factor	mES	EpiSC	hPSC	hEC
LIF	+	-	-	-
BMP4	+	-	-	-
Activin A and/or Nodal	-	+	+	-
FGF 2	-	+	+	-
Stem cell surface markers				
SSEA-1	+	+	-	-
SSEA-3	-	+	+	+
SSEA-4	-	+	+	+
Tra 1-60	+	+	+	+
Tra 1-81	+	+	+	+
GCMT 2		+	+	+
H-type 1	NE	NE	+	NE
Tra 2-49/alkaline phosphatase	+	+	+	+
Stem cell associated genes				
OCT4	+	+	+	+
NANOG	+	+	+	+
SOX2	+	+	+	+
KLF4	+	-	+	+
REX1		-	+	+
Other features				
Telomerase activity	+	+	+	+
X-inactivation	-	+	+	+
Developmental potential				
Teratoma formation	+	+	+	+
Chimera formation	+	-	NE	

+ the gene or surface marker is expressed/the indicated growth factors are required for self renewal/X-inactivations occur in the indicated cell type/the indicated cell type will form teratomas or participate in chimera formation.

- the gene or surface marker is not expressed/the indicated growth factors are not required for self renewal/X-inactivations do not occur in the indicated cell type/the indicated cell type will not form teratomas or participate in chimera formation.

NE the attribute is not examinated

2.5 APPLICATION OF HUMAN PLURIPOTENT STEM CELLS

The pluripotent stem cell field is full of possibilities. Human ES cells provide a unique model to study human embryonic developmental events that cannot be studied directly in the intact human embryos. A very significant advantage of the hiPS cells compared to hESC, is their potential to generate patient-specific cells especially for disease modelling but also autologous cell transplantation. Due to their proliferation capacity and ability to differentiate into any cell types in the body,

hPSCs have gained a lot of attention as well. Major efforts worldwide are currently focused on the application of stem cells in drug and toxicological research and disease modelling (Silva et al. 2008; Carvajal-Vergara et al. 2010; Moretti et al. 2010; Hockemeyer et al. 2011; Najm et al. 2011; Hasehira et al. 2012; Lahti et al. 2012). However, there are still many challenges to solve, particularly related to technical, regulatory and clinical questions.

2.5.1 Therapeutic promises

The world's first, and only, clinical trial testing a hESC therapy (oligodendrocyte progenitor cells derived from hESCs for spinal cord injury; Geron Corporation www.geron.com) was terminated in 2011 because of the economics, not for the lack of promise. However, the first Phase I clinical trials using hESC-based cellular products for eye diseases have recently been approved by the regulatory authorities in the USA, EU and Japan (http://stemcells.nih.gov/info/health.asp) raising hopes that at least some of the therapeutic promises of hPSCs will gradually be realised.

Manipulation of the hPSC genome has become a routine in the last few years. The targeted and controlled genetic engineering with nuclease-based methods play now an essential role in evaluating the effects of gene defects in diseases (Hockemeyer et al. 2011; Sebastiano et al. 2011; Zou et al. 2011; Wang et al. 2012), and it could open the way to completely new approaches in regenerative medicine as well.

2.5.1.1 Teratogenity

In iPSC technology, the reintroduction of "stemness genes" can have undesirable adverse consequences. A close relationship between pluripotency and tumorigenity includes the risk of tumor formation from an undifferentiated population of the transplanted cells or from an unstable cell population that can de-differentiate or transform the cells to produce cancerous cells (Carpenter et al. 2009; Knoepfler 2009; Krizhanovsky and Lowe 2009; Wobus 2010). For instance, the potential for reactivation of introduced genes such as c-myc and Oct4 can increase the risk of cancer. These issues will need to be addressed before iPS technology can be transferred into clinics.

The tumorigenity of pluripotent cells will be eventually overcome by direct lineage reprogramming of somatic cells (Ieda et al. 2010; Vierbuchen et al. 2010; Ambasudhan et al. 2011; Pang et al. 2011; Buganim, et al. 2012). This

reprogramming method could avoid pluripotent, potentially tumorigenic state and directly generate induced differentiated cells. Further studies are needed to understand consequences of direct lineage reprogramming strategies.

3. AIMS OF THE STUDY

The aims of this thesis were to:

- ✓ investigate the capacity of human foreskin fibroblasts to support human embryonic stem cell self- renewal.

- ✓ characterize new Finnish hESC lines and investigate their developmental capacity.

- ✓ identify the N-glycan profile of human embryonic stem cells and find potential molecular markers to specify the stage of differentiation of hESCs.

- ✓ develop and evaluate a single component, animal-free cell culture matrix for human pluripotent stem cells.

4. MATERIALS AND METHODS

All methods used in this thesis project are briefly described here and in detail in the "material and methods" sections of the original articles.

4.1 Ethical approval

An informed consent form for embryo donation was signed by both partners after receiving an oral and written description of the study. The donors did not receive financial compensation. Donated embryos were received from Fertility Unit at Karolinska University Hospital, Huddinge, Sweden. Only embryos not suitable for embryo transfer or cryopreservation were cultured to blastocyst stage and used for derivation of hESC lines (publication I).
In paper II, donated embryos were received from Fertility Clinic, Väestöliitto, Helsinki (publication II) and only frozen leftover embryos were used. The human embryonic stem cell lines used in this study were derived in University of Helsinki, and this study was approved by the ethical board of the Helsinki and Uusimaa district hospital (HUS).
Human iPS cells were derived at the University of Helsinki, with the permission of the ethical board of the Helsinki and Uusimaa district hospital (HUS) (publication IV).

4.2 Derivation of embryonic stem cell lines

The isolation of the inner cell masses from blastocysts was performed using immunosurgery. The zona pellucida was removed using 0.5% pronase (Sigma-Aldrich Co. USA) and trophectoderm was then removed by immunosurgery (Solter and Knowles 1975; Thomson, Itskovitz-Eldor et al. 1998) The blastocyst was incubated with rabbit antihuman whole serum (Sigma) and guinea pig complement serum (Sigma), resulting in lysis of the trophectoderm.

4.3 Human induced pluripotent stem cells

Two different human iPS cell lines (FiPS 5-7 and HEL11.4) were used. FiPS 5-7 was generated from human foreskin fibroblasts (CRL-2429; ATCC, Manassas, VA, USA) using retrovirus-induced overexpression of OCT4, SOX2, NANOG, and LIN-28 (Hussein, Batada et al. 2011).
HEL11.4 was generated from adult fibroblasts (male, 84 years old) using retrovirus-induced overexpression of four genes: OCT4, SOX2, KLF4, and c-Myc. Cells were

infected with virus-containing supernatant twice at 24-h intervals. The infected cells were harvested and re-seeded on mitotically inactivated treated MEF layer three days after infection. Twenty to thirty days post-transduction ES-like colonies were picked, expanded, and characterized.

4.4 Feeder cells

4.4.1 Human feeder cells

Human foreskin fibroblasts (hFF) (CRL-2429; ATCC, Mananas, VA, USA) were used as feeder cells. They were mitotically inactivated either by irradiation (40Gy) (publication I) or by Mitomycin C (Gibco, Life Technologies) (publications II and III). Approximately 15 000 cells/cm^2 were plated onto dishes (NUNC or Corning) and left overnight to form a confluent monolayer.

4.4.2 Mouse feeder cells

ICR-strain mouse embryonic fibroblast cells (embryonic day 12.5-13.5) were used. They were mitotically inactivated by Mitomycin C (Gibco, Life Technologies), approximately 20 000 cells/cm^2 were plated onto dishes (NUNC or Corning) and left overnight to form a confluent monolayer.

4.5 Extracellular matrix coating

4.5.1 Matrigel™

Plates coated with Matrigel™ (BD Biosciences, Bedford, MA) were prepared according to the manufacturer's instructions by diluting Matrigel™ 1:200 in cold DMEM/F12 (Life Technologies) followed by incubation of the culture plates with diluted Matrigel™ at RT 2 hours or 4 ºC until used. The Matrigel™-coated plates were equilibrated for one hour in room temperature before use, and excess Matrigel™ was removed and the plate was washed once with DMEM/F12 just before adding the cell suspension.

4.5.2 ECA

ECA (1mg/ml) was thawed slowly at 4ºC and diluted 1:15 in PBS. It was plated at 5µg/cm^2. The plate was incubated at 4°C overnight. Next day, the plate was washed twice with PBS and once with DMEM/F12, before adding the cell suspension.

4.6 Culturing human pluripotent stem cells

4.6.1 Culture medium

Human and mouse feeder cells were cultured in DMEM with Glutamax (Gibco) supplemented with 10% FBS (Promocell). Media was changed to KO-DMEM media before transfer of hPSCs onto feeder cells. The medium used for culture and derivation of the stem cells on feeder cells (KO-DMEM media) consisted of Knockout Dulbecco's Modified Eagle's Medium or DMEM/F12 supplemented (20%) with Knockout SR, 2 mM Glutamax, 1% non-essential amino acids (all from Gibco, Life Technologies), 0.1mM 2-mercaptoethanol (Sigma-Aldrich) and bFGF (Biosource/ Life Technologies) at 6 ng/ml.

To prepare mEF-conditioned media (CM-media), mEF cells were cultured in T150 bottles and when 80-90% confluent MEF media was changed to KO-DMEM media. Media was collected once a day during five days, filtered, supplemented with 8ng/ml bFGF and frozen until use.

Commercially available culture media StemPro (Life Technologies) and mTeSR1 (Stem Cell Technologies, VA, USA) were used together with Matrigel^{TM} (BD) or ECA according to manufacturer's instruction.

4.6.2 Passaging

In early passages hPSCs were passaged mechanically by slicing small pieces of the colonies with a glass capillary, a surgical scalpel or a needle, and then transferred onto a new plate using a pipette. In publication I dispase solution (10mg/ml, GibcoBRL, Life Technologies) was used before slicing the colonies to loosen cells from feeder cells. With this passaging method the hPSCs can be expanded 1:2-1:5 with a five to ten day interval depending on the cell line and passage number.

Enzymatic passaging was performed by collagenase IV treatment (Gibco, Life Technologies). The culture plates were incubated with collagenase IV for three to seven minutes at 37 ºC until the edges of the colonies started to curl up. After incubation, collagenase IV was removed, the plate were gently washed twice with DMEM/F12 before adding fresh culture media and scraping of the colonies in small aggregates and transferred onto feeder- or matrix-coated plates (publication II, III, IV).

In single cell assays (publication IV) cells were dissociated using TrypleSelect (Gibco, Life Technologies) according to manufacturer's description and cells were counted with haemocytometer.

4.6.3 Cryopreservation of hPSCs

Two different methods have been used in cryopreservation. Early hESC lines were frozen by a vitrification method in open pulled straws (LEC instruments Pty. Ltd, Australia) (Reubinoff et al. 2001). The undifferentiated colonies were mechanically cut into small pieces using a surgical scalpel or a needle and washed in cell culture medium. Four to eight colony pieces were transferred in Vitrification media 1 (DMEM that contained HEPES buffer supplemented with 20% FBS, 10% DMSO and 10% ethylene glycol) and incubated for 1 minute, followed by 25 seconds of incubation in Vitrification media 2 (DMEM that contained HEPES buffer supplemented with 20% FBS, DMSO and ethylene glycol, and 0.5 mol/l sucrose). The colony pieces were then transferred into a droplet of 5µl of Vitrification media 2 from where the colonies where loaded into the straw. The straw was immediately submerged into liquid nitrogen for long-term storage.

In slow freezing method for larger quantities, the cells were cut out from plates either mechanically or enzymatically, washed once in cell culture medium and centrifuged 400 rpm for 4 minutes. The cell pellet was gently resuspended into Freezing media1 (KO-DMEM supplemented with 20% FBS). Equal amount of cold Freezing media 2 (Freezing media 1 with 20% DMSO) was added drop wise and cell suspension were moved into the cryo-vial and stored at -70% o/n. On the next day cryo-vials were moved into -150 °C via liquid nitrogen.

4.7 Characterization

4.7.1 Flow Cytometry analysis (FACS)

Single-cell suspensions of hPSCs were obtained by dissociation with TrypLE (Life Technologies) 5 min at 37°C, washed twice with cold FACS buffer (PBS supplemented with 5% FBS) and cell suspension was passed through an 80 µm cell strainer (BD Biosciences). Cells were incubated with primary antibodies (SSEA3, Tra 1-60, H-type1 and SSEA1) (Table 4) on ice for one hour, washed once with cold FACS buffer and probed with a secondary antibody for 30 min in dark on ice and washed once. Control cells were incubated only with secondary antibody. The used

antibodies are listed in Table 4. Cells were analysed in a FACSCalibur or FASCAria flow cytometer and analysed with Cell Quest software (all from Becton Dickinson).

Table 4. Antibodies used in characterization analysis

Primary antibody	**Manufacturer**	**Secondary antibody**
SSEA-3	Millipore, Invitrogen, Stemgent	Anti-Rat IgG
Tra 1-60	Millipore, Invitrogen	Anti-Mouse IgM
H-type1	Abcam	Anti-Mouse IgG
SSEA- 1	Millipore	Anti-Mouse IgM
Oct4	Santa-Cruz	Anti-Rabbit IgG
Nanog	Cell Signalling	Anti-Rabbit IgG
Sox2	Cell Signalling	Anti-Rabbit IgG
E-cadherin	Cell Signalling	Anti-Mouse IgG
FOXA2 / HNF3beta (P-19)	Molecular Probes, Invitrogen	Anti-Goat IgG
Anti-Human Alpha-1-Fetoprotein	Molecular Probes, Invitrogen	Anti-Rabbit IgG
Anti-Human/Mouse Serum Albumin	Molecular Probes, Invitrogen	Anti-Mouse IgG
CXCR4	BD Bioscience	Anti-Rabbit IgG

4.7.2 Immunocytochemical Analysis

hPSCs cultured on feeders, on Matrigel^{TM} or on ECA-coated wells were rinsed once with PBS before fixation with 4% paraformaldehyde for 15 min at RT. For staining of nuclear markers (Oct4, Nanog, Sox2) and E-cadherin, cells were permeablized with Triton-X for 30 min at RT.

Cells were blocked either with appropriate 4% serum (publication I & II) or with Ultra-V Block (Thermo Scientific) (publication IV) for 10 min at room temperature. Primary antibodies were incubated at 4°C for overnight, washed three times with PBS, and secondary antibodies were incubated at room temperature for 1 hr. Wells were then washed with PBS and counterstained using Vectashield Mounting Medium with DAPI (Vector Labs). The used antibodies are listed in Table 4.

4.7.3 Lectin Analysis

In publication III cells were stained using lectins. The cells were incubated with fluorescein-labeled lectins for one hour at room temperature, washed twice with PBS and counterstained using Vectashield Mounting Medium with DAPI (Vector Labs). Specific known inhibitors were used to control the specificity of lectin binding. Cells were incubated with the inhibitor for one hour, washed once and stained with lectins. The tested lectins and their inhibitors are listed in Table 5.

Table 5. The lectins and inhibitors

Lectin	Manufacturer	Inhibitor	Manufacturer
Maackia amurensis, *MAA*	EY Laboratories	α3'-sialyllactose	Kyowa Hakko Kogyo, Japan
Pisum sativum, *PSA*	EY Laboratories	α-D-mannose methyl glycoside	Sigma-Aldrich
Ulex europaeus, *UEA-I*	EY Laboratories	L-fucose	Danisco Sweeteners, Finland

4.7.4 RNA isolation, reverse transcription, RT-PCR and quantitative PCR

Total RNA was extracted from cells using RNA isolation kit (either SV Total RNA system from Promega, NucleoSpin RNA II from Macherey & Nagel or Qiagen RNeasy Mini kit) according to manufacturer's instructions. Total RNA was calculated using a Nano-Drop spectrophotometer and cDNA was synthesized from 0.5-2µg of total RNA.

In RT-PCR 100ng of cDNA was used for PCR amplification with AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA). Primers used are listed in Table 6.

Table 6. RT PCR primers used.

Primer	Sequence of 5'-primer	Sequence of 3'-primer	Product size
NANOG	GGAAGACAAGGTCCCAGTCA	ATTGTTCCAGGTCTGGTTGC	349 bp
OCT4	CGTGAAGCTGGAGAAGGAGAAGCTG	AAGGGCCGCAGCTTACACATGTTC	245 bp
FGF4	TCACCGATGAGTGCACGTTCA	GAGGAAGTGGGTGACCTTCAT	158 bp
STELLA	CACAAATGCTCACCGAAGAA	TTCGATTTCCCTGAGGACTG	182bp
TEX14	TCCTGTTTTTGGAAGCGACT	GTGGCAGCTGAACAAAGTGA	214 bp
PIWIL2	TCTATGGGGCCATCAAGAAG	CCATCCCGATCACCATTAAC	195 bp
DAZL	GGAGCTATGTTGTACCTCC	CCATGTAACTAGATAAGCCAG	313 bp
PUM2	CCAACATTCCTTGGTGAG	ATCAGGACCCCAAGAAGAGG	402 bp
C-ACTIN	TGATATCCGCAAGGACCTGT	GCTGGAAGGTGGACAGAGAG	200 bp
MYOSIN	TCCATGTTCGACCAGACTCA	AAGCGGTCACACTGCGTGGT	335 bp
CMP	AGAGCTACAGCGTCATCGAG	TCTGACACGTCCAGCGTATC	316 bp
NF-L	CAAAGAGTGAAATGGCACGA	AGCGGGTGGACATCAGATAG	231 bp
TUBULIN3β	CATCCAGAGCAAGAACAGCA	TCGGTGAACTCCATCTCGTC	234 bp
ALBUMIN	GCACAATGAAGTGGGTAACC	CAGCAGTCAGCCATTTCACC	349 bp
FOXA2	TGCCAGGAGCACAAGCGAGG	TGTTCGTAGGCCTTGAGGTCC	290 bp
HNF1α	CAGGTCTTCACCTCAGACAC	GAGGCCATCTGGGTGGAGAT	263 bp
HNF1β	ACCTTGACGAATATCCACAGC	CTGTGACCACCATTGCAGATG	364 bp
HNF6	AGGGCAGATGGAAGAGATCA	TGGATGGACGCTTATTTTCC	377 bp
PDX1	ACCAAAGCTCACGCGTGGAA	CTCTCGGTCAAGTTCAACAT	191 bp
GAPDH	GTCTTCACCACCATGGAGAAGGCT	TGTAGCCCAGGATGCCCTTGAGGG	529 bp

In qPCR 50ng of cDNA was used with SYBR Green PCR Master Mix (Sigma Aldrich). Quantitative PCR was performed on an Mx3000P qPCR system (Stratagene) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 20s, 56°C for 25s and 72°C for 20s. The sequences of the primers used

in qPCR are listed in Table 7. The expression levels of each gene were normalized to Cyclophilin and the relative quantification was performed using the comparative C_T method. All PCR amplifications (both RT-PCR and qPCR) were performed in duplicate and replicated in at least two independent experiments.

Table 7. Quantitative PCR primers used.

Primer	**Sequence of 5'-primer**	**Sequence of 3'primer**
OCT4	TTGGGCTCGAGAAGGATGTG	GTGAAGTGAGGGCTCCCATA
NANOG	GAACAATCAGGCCTGGAACAGT	CGCTGATTAGGCTCCAACC
SOX2	ACACTGCCCCTCTCACACAT	TCTCTTTTGCAGCTGTCATTTG
GOOSECOID	GAGAACCTCTTCCAGGAGAC	TTCTTAAACCAGACCTCCAC
BRACHUYRY	GCATGATCACCAGCCACTG	TTAAGAGCTGTGATCTCCTC
AFP	CGCTGCAAACGATGAAGCAAG	AATCTGCAATGACAGCCTCAAG
Albumin	GGAAAAGTGGGCAGCAAATGT	GGTTCAGGACCACGGATAGA
CER1	CATTGGGAGACCTGCAGGAC	CCCAAAGCAAAGGTTGTTCTG
FoxA2 (HNF-3ß)	AAGACCTACAGGCGCAGCT	CATCTTGTTGGGGCTCTGC
Cyclophilin	CAATGGCCAACAGAGGGAAG	CCAAAAACAACATGATGCCA

In PCR array analysis, RNA was isolated using Qiagen RNeasy Mini kit; cDNA was synthesized using RT² First Strand Kits (Qiagen). The cDNA was characterized on the ABI PRISM® 7000 Real-Time PCR System (Applied Biosystems) using two different RT2 Profiler PCR Arrays (SABioscience): Embryonic stem cells (PAHS-081) (Table 8a) and Extracellular matrix and adhesion molecules (PAHS-013) (Table 8b). The resulting raw data were then analysed using the PCR Array Data Analysis Template.

Table 8a. List of 84 genes of embryonic stem cell PCR-array (SABioscience)

	Genes/markers
Transcription Factors Maintaining "Stem-ness"	FOXD3, GATA6, GBX2, NANOG, NR5A2, NR6A1, POU5F1, SOX2, TFCP2L1, UTF1, ZFP42.
Signaling Molecules Required for Pluripotency and Self-Renewal	COMMD3, CRABP2, EDNRB, FGF4, FGF5, GABRB3, GAL, GRB7, HCK, IFITM1, IL6ST, KIT, LEFTY1, LEFTY2, LIFR, NODAL, NOG, NUMB, PTEN, SFRP2, TDGF1.
Cytokines and Growth Factors	FGF4, FGF5, GDF3, LEFTY1, LEFTY2, NODAL, TDGF1.
Other Embryonic Stem Cell-Specific Genes	BRIX1, CD9, DIAPH2, DNMT3B, IFITM2, IGF2BP2, LIN28A, PODXL, REST, SEMA3A, TERT.
Selected Embryonic Stem Cell Differentiation / Lineage Markers	FOXA2, GATA4, PTF1A (Extra-Embryonic Endoderm) CDX2, EOMES, GCM1, KRT1(Trophoblast) AFP, SERPINA1(Visceral Endoderm) FN1, LAMA1, LAMB1, LAMC1, SOX17 (Parietal Endoderm T, WT1 (Mesoderm) DES, MYF5, MYOD1(Muscle) HBB, HBZ (Blood) COL1A1, RUNX2 (Bone) NES, NEUROD1, PAX6 (Neural) CD34, CDH5, FLT1, PECAM1(Endothelial) DDX4, SYCP3 (Germ Cell) GCG, IAPP, INS, PAX4, PDX1, SST(Pancreas)
Cell-Differentiation / Lineage Markers	OLIG2, TAT

Table 8b. List of 84 genes of extracellular matrix and adhesion molecules PCR array (SABioscience).

	Genes
Cell Adhesion Molecules	CD44, CDH1, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, MMP14, MMP15, MMP16, NCAM1, PECAM1, SELE, SELL, SELP, SGCE, SPG7, VCAM1 (Transmembrane Molecules) CD44, CDH1, COL11A1, COL14A1, COL6A2, CTNND1, ICAM1, ITGA8, VCAM1 (Cell-Cell Adhesion) ADAMTS13, CD44, ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, SGCE, SPP1, THBS3 (Cell-Matrix Adhesion) CNTN1, COL12A1, COL15A1, COL16A1, COL5A1, COL6A1, COL7A1, COL8A1, VCAN, CTGF, CTNNA1, CTNNB1, CTNND2, FN1, KAL1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, THBS1, THBS2, CLEC3B, TNC, VTN (Other Adhesion Molecules)
Extracellular Matrix Proteins	COL4A2, COL7A1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, SPARC (Basement Membrane Constituents) COL11A1, COL12A1, COL14A1, COL15A1, COL16A1, COL1A1, COL4A2, COL5A1, COL6A1, COL6A2, COL7A1, COL8A1, FN1, KAL1 (Collagens & ECM Structural Constituents) ADAMTS1, ADAMTS13, ADAMTS8, MMP1, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP3, MMP7, MMP8, MMP9, SPG7, TIMP1 (ECM Proteases) COL7A1, KAL1, THBS1, TIMP1, TIMP2, TIMP3 (ECM Protease Inhibitors) VCAN, CTGF, ECM1, HAS1, SPP1, TGFBI, THBS2, THBS3, CLEC3B, TNC, VTN (Other ECM Molecules)

4.8 Pluripotency assays

4.8.1 Embryoid body formation

Briefly, on the day of passage, the undifferentiated hPSCs were treated with collagenase IV for 5 min and then transferred to 6-well ultra-low attachment plate (Corning). By culturing aggregates of hPSCs in suspension without bFGF for 7-10 days, embryoid bodies (EBs) were formed. The presence of tissue originating from the three embryonic germ layers was demonstrated using immunohistochemistry for antigens (Table 9) of the three embryonic germ layers.

Table 9. Markers used in teratoma analysis

Antigen	**Germ layer/Structure**
Vimentin	Mesoderm /Mesenchyme
Desmin	Mesoderm /Muscle
Neurofilament	Ectoderm/Neuron
HNF3β	Endoderm
CK-19	Epithelium
Ki-67	Cell proliferation

4.8.2 EB derivatives, stage 3 cells

Embryoid bodies were transferred onto gelatin-coated cell culture plates, cultured ten days in DMEM/F12 supplemented with Insulin (1mg/ml)-transferrin (0.55mg/ml)-Sodium Selenite (0,5 µg/ml) (ITS), Fibronectin (Sigma), L-glutamine and Penicillin-Streptomysin.

4.8.3 Teratoma formation

To evaluate pluripotency and study the developmental potency *in vivo*, we used severe combined immunodeficient (SCID)-beige mice (publication I) and nude mice (publications II, IV). Nude mice are homozygous for a mutation in the forkhead box N1 gene that causes hairlessness and impaired thymus development, resulting in immature T cells and associated immunodeficiency.

In brief, exponentially growing hPSC lines were harvested from the culture plates using enzymatic splitting. Cells were washed twice in DMEM/F12 and put into a 1.5-ml collection tube containing 30 µl of culture medium. Approximately 10^3 to 10^4 cells were implanted under the testicular capsule. Animals were anesthetized by a mixture of Ketamine and Xylatsine, and Carprofen was used as painkiller during the

operation and day after. Animals were housed under controlled humidity, temperature, and light regimen, and care was consistent with institutional and National Institute of Health guidelines.

Teratoma growth was followed by palpation, and after seven to eight weeks, the mice were sacrificed using CO_2 and the teratoma was dissected out. The presence of tissue components of all three embryonic germ cell layers was analysed from the haematoxylin-eosin stained sections by routine histological analysis or using immunohistochemistry for marker genes (neurofilament, desmin, HNF3β) of the three embryonic germ layers.

4.9 N-glycan profiling

hESCs and EBs were collected mechanically, washed and stored frozen until analyses. The total N-glycan pool was separated by an ion-exchange step into neutral N-glycans and acidic N-glycans, and these two glycan fractions were analysed separately by MALDI–TOF mass spectrometry (Figure 10). This gives a global view of the N-glycan repertoire and allows comparative analysis of differentiation-associated changes. Detailed structural analyses were achieved from the total neutral and acidic N-glycan pools by a combination of proton NMR spectroscopy, specific glycosidase digestions, and MS/MS fragmentation experiments. The glycome profile data was used to design glycan-specific labeling reagents. The most interesting glycan types were chosen to study their expression profiles by lectin histochemistry.

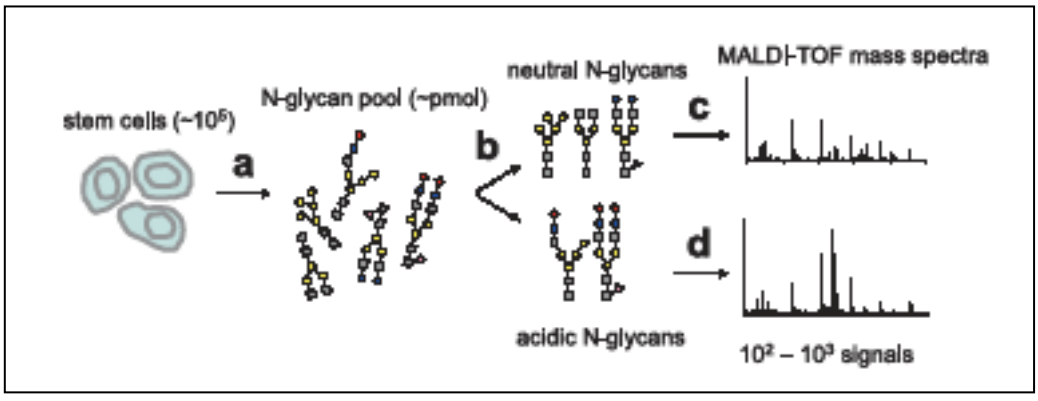

Figure 10. The N-glycan profiling. A. Total N-glycan pool was enzymatically isolated from about 100.000 cells. b. The total N-glycan pool was purified with microscale solid-phase extraction and divided into neutral and sialylated N-glycan fractions. The N-glycan fractions were analyzed by MALDI-TOF mass spectometry either in positive ion mode for neutral glycans (C) or in negative ion mode for sialylated glycans (D). Over one hundred N-glycan signals were detected from each cell type. The relative abundances of the observed glycan signals were determined based on relative signal intensities (Saarinen et al. 1999).

4.10 Population growth curve and viability

In publication II, cells were dissociated with Trypsin-EDTA and 10 000 to 40 000 cells were plated on 24-well plates. Thereafter, cells were counted using Hemocytometer at two time points, 24h and 48h after plating.

In publication IV, cells were dissociated first by collagenase IV as described earlier and subsequently, an aliquot of these cells was dissociated into single cells by TrypleSelect. Approximately 6000 cells were plated onto a 12-well plate. Cell number was counted at day 3 and day 6 time points. Cells were counted using Hemocytometer. The viability of the dissociated cells was analysed using CellCounter (Invitrogen) and statistical significance was determined using student's two-tailed *t*-test.

4.11 Clonogenic assay

Undifferentiated hPSCs were treated with TrypLE Select for 5 min at +37C to dissociate them into single cells and passed through an 80 µm cell strainer (BD).
Cells were plated at cell density 35cells/cm^2 onto *ECA* or Matrigel™ (BD). Media was changed every other day. After ten days, the number of colonies was counted.

4.12 Colony area measurement

Cells were dissociated as described above (clonogenic assay doubling) and plated (600 cells/cm^2) onto *ECA* or Matrigel™ (BD). The plates were then imaged using Cell IQ (CM technologies) every fifth hour. After 6 days the images were analysed using CellIQ Analyser program (CM technologies).

4.13 Validation of binding specificity

Undifferentiated hPSCs were passaged enzymatically by collagenase IV (Gibco, Life Technologies) and plated 1:2 on ECA coated 12-well plates. Lactose monohydrate ($LacH_2O$) (Sigma-Aldrich, 100mM), lacto-N-neoteraose (LnNT) (Kyowa Hakko Kogyo, 100mM) and saccharose (Sigma-Aldrich, 100mM), were added to culture media during passaging. The attached cells were counted after 20 hours.

4.14 Targeted differentiation

Human embryonic stem cell lines FES29 and H9, and iPS cell line HEL11.4 were cultured on ECA and Matrigel^{TM} in mEF-conditioned medium (CM) supplemented with bFGF. When the cultures reached 80% confluence, the differentiation experiments were initiated.

A modified three-step differentiation protocol for hepatocyte differentiation was used (Hay, Zhao et al. 2008). Details of the protocol are illustrated in Table 10.

Table 10. Hepatocyte differentiation protocol.

Stage 1. → The first day of DE induction	**Definitive endoderm differentiation**	**Stage 2. Differentiation to Hepatic progenitors**	**Stage 3. Hepatocyte maturation**
RPMI1640 +Glutamax B27 2% (v/v) Wnt3a 75 ng/ml ActA 100 ng/ml NaB 1mM	RPMI1640 +Glutamax B27 2% (v/v) ActA 100 ng/ml NaB 0.5 mM	KO-DMEM KO-SR 20% (v/v) NEAA 1% (v/v) Glutamine 1mM βMeOH 0.1mM DMSO 1% (v/v)	Leibovitz's L15 FBS 8.2% (v/v) Tryptose phosphate broth 8.3% (v/v) Hydro-cortisone-21-hemisuccinate 10µM Insulin 1µM Glutamine 2mM HGF 10ng/ml OncM 20ng/ml
1 day	4 days	5 days	10 days

DE= definitive endoderm, ActA= Activin A, NaB= Natrium butyrate, NEAA= Non essential aminoacids, DMSO=dimethyl sulfoxide, HGF=hepacyte growth factor, OncM= Oncostatin M

4.15 Statistical analysis

Statistical analysis were performed using a two-tailed t-test or the analysis of one-way analysis of variance (ANOVA) by the SPSS Statistics program, followed by Tukeys post hoc test. In publication III, the statistical analysis of glycan score distribution were performed using a two-tailed t-test with Welch's approximation and a two-tailed Mann-Whitney U test. All p-values less than 0.05 were regarded as statistically significant. The results are expressed as ± SD or SEM.

5. RESULTS AND DISCUSSION

5.1 PUBLICATION I: HUMAN FORESKIN FIBROBLASTS AS FEEDER CELLS IN HUMAN EBMRYONIC STEM CELL

Finding the optimal xeno-free techniques to culture hESCs is important for possible future cell therapies. The aim of our study was to avoid mouse feeder cells in human embryonic stem cell culture and to find a simple and robust animal-free culture system. In this study we used commercially available postnatal human fibroblasts, derived from foreskin.

Inner cell mass was isolated from five human blastocysts, and two lines, HS181 and HS207, were established. The donated, leftover blastocysts were evaluated using a scoring system based on blastocyst expansion, inner cell mass, and trophectoderm development (Gardner and Schoolcraft 1999). None of the blastocysts was of top quality. Stem cell line HS 181 was derived from expanded blastocyst with a small ICM and small cell number in trophectoderm (score 4BB). HS 207 was derived from one of the most poorly developed blastocyst used in the study; it was not expanded at all, the ICM was practically invisible and the cell number in trophectoderm was small (score 2CB). These results indicate that the blastocyst morphology does not directly predict the outcome of the derivation. However, more blastocysts would have been needed to establish the relationship between blastocyst quality and hESC line derivation efficacy (Strom et al. 2010).

Both cell lines formed morphologically normal tightly packed colonies. HS 181 was cultured over nine months on hFF and was fully characterized while the characterization of HS 207 was only partial when the study was submitted for publication. HS181 cells expressed the pluripotency markers Oct4, Tra 1-60 and SSEA-4 as well as alkaline phosphatase, and it also maintained normal 46, XX karyotype. The capacity of differentiation in *in vivo* was demonstrated by the teratoma assay. Cells were cultured for 20 passages on hFF and then injected under the testicular capsule of SCID/beige mice. Resulting teratomas were analysed and they were found to contain tumour-like structures and cells from all three embryonic layers.

The use of non-human materials bears a risk of transmitting animal derived pathogens so the use of human cells would be preferable over the use of mouse cells

as feeders. Before this study, different human cell types like adult tubal epithelial cells and foetal muscle and skin cells had been successfully used as feeder cells (Richards et al. 2002). Obtaining those cell types is not simple. Commercially available human foreskin fibroblasts offer a simple way for derivation and continued undifferentiated human embryonic stem cell growth without xenogenic contamination. The result of this study was a step forward towards clinical grade stem cells. However, several commercially available feeder-free culture systems now exist (StemPro and Essential 8 (http://www.lifetechnologies.com), mTeSR™1, mTeSR™2 and TeSR™-E8™ (http://www.stemcell.com), NutriStem XF/FF (www.stemgent.com), Pluripro® (http://www.cellgs.com)) offering defined conditions for culturing even clinical grade hESCs and hiPSCs.

5.2 PUBLICATION II: DICTINCT DIFFERENTIATION OF INDIVIDUAL HUMAN EMBRYONIC STEM CELL LINES

Individual cell lines may have distinct specific genetic and/or epigenetic profiles that may affect their differentiation properties (Hoffman and Carpenter 2005; Hoffman et al. 2005; Lagarkova et al. 2006). The first aim of study II was to focus on the differentiation characteristics between five new Finnish embryonic stem cells lines. Secondly, we analyzed the mRNA expression of primordial germ cell associated genes in these hESC lines to test the hypothesis that embryonic stem cells might represent early committed primordial germ cells (PGCs) rather than epiblastic cells (Matsui and Okamura 2005; Zwaka and Thomson 2005).

Out of 323 thawed zygotes only 83 developed to blastocyst stage and 70 inner cell masses were successfully isolated. The blastocysts were evaluated using a previously described scoring system (Gardner and Schoolcraft 1999). We derived five stable pluripotent hESC lines (FES 21, 22, 29, 30 and FES61). One early line (FES 11) was lost between passages 11 and 15. This gave a derivation success rate of 7%.

Two of the lines (FES 21 and FES 22) were established on mouse feeder cells and three (FES 29, FES30 and FES 61) on human foreskin fibroblast, but all hESC lines were later cultured and analysed on both mouse and human feeder cells. We did not find any differences in cell or colony morphology between cell lines or between different types of feeder cells when the cells were passaged mechanically. Cell lines also expressed all the tested pluripotency markers including transcription factors OCT4 and NANOG and cell surface markers Tra 1-60, Tra 1-81 and SSEA-4 as well

as alkaline phosphatase both on mEF and hFF. However, depending on feeder type, we found differences in the growth rate and density of colonies. Population doubling time of hESC was 1,5 times higher when cultured on mFF as compared to hEF (Figure 11).

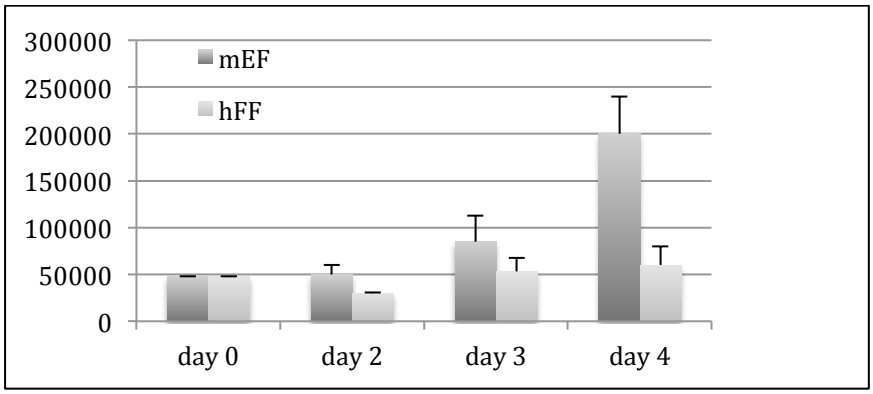

Figure 11. Population doubling time on mEF and hFF. FES 22 cells proliferate faster on mouse feeder cells than on human feeder cells (mean ± SD).

Also the density of hESC colonies was much higher on mEF than on hFF and smaller cell clusters survived on mEF when passaging cells mechanically. When using enzymatic passaging, human feeder cells did not support long-term pluripotent cell growth.

The karyotype analysis showed that culture conditions or techniques may affect chromosomal stability. When cells were passaged mechanically on hFFs, they maintained their normal karyotype in long-term culture whereas enzymatic passaging on mEF increased the likelihood of an abnormal karyotype. Changes were most often seen in chromosomes 12 and 17. Our results indicate that culture conditions can have significant effects on many characteristics of hESCs, like growth rate and genetic stability, but it remained unclear what parameters, or combination of parameters mostly influence these findings. The International Stem Cell Initiative Consortium (ISCI) analyzed 125 hESC lines (including also FES 21, 22, 29 and 30) and 11 induced pluripotent stem (iPS) cell lines, from 38 laboratories worldwide at early and late passages (Adewumi et al. 2007; Amps et al. 2011). The tendency to chromosomal abnormalities grew during prolonged culture. The frequent chromosomal changes occurred in chromosomes 1, 12, 17 and 20, representing mostly non-random chromosomal gains. This suggests that these chromosomes might include genes, which provide growth advance for the undifferentiated cells. The results indicated also that enzymatic mass-passaging

techniques may favor the generation of abnormalities during prolonged culture relative to cells passaged manually.

The embryoid bodies and teratomas from established cell lines showed distinct differentiation propensities. FES 30 cell line produced significantly less EBs during seven days than other cell lines (Figure 12). They were also less differentiated, showing persistence of the pluripotency marker OCT4 and lower expression of the tested lineage-specific markers.

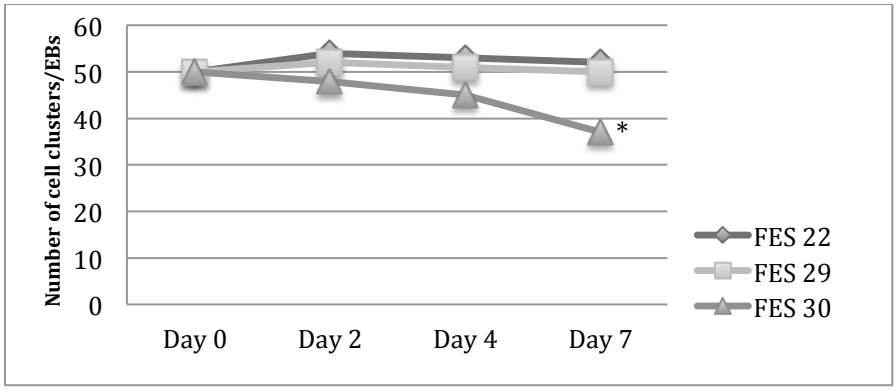

Figure 12. Capacity of EB formation between cell lines. Fifty pieces of colonies were placed in EB suspension culture and the number of developing EBs counted during seven days. FES 22 and FES 29 lines formed more EBs as compared to FES 30 ($p<0.001$ at day 7, mean SD, $n = 7$).

Likewise, the teratomas derived from FES 30 were poorly developed as compared to the other four cell lines. Teratomas from FES 30 consisted mostly of cystic structures when other cell lines formed solid teratomas. The gene expression and histology analysis proved that FES 30 teratomas were characteristically immature while other cell lines produced teratomas with multi-lineage tissue.

We had previously made genetic profiling for FES 21, 22, 29 and 30, and they were not remarkably different from each other (Skottman et al. 2005) making the difference in functional tests unexpected. Interestingly, FES 30 is the only female line and epigenetic regulation of the X chromosome in females constitutes an important mechanism for gene dosage compensation (Chang et al. 2006). hESCs are known to vary in their potential to undergo X chromosome inactivation; some female lines express detectable levels of X-inactive specific transcript (XIST) whereas other lines express low or undetectable level of XIST (Hoffman et al. 2005; Adewumi et al. 2007; Dvash et al. 2010; Lengner et al. 2010; Anguera et al. 2012). The loss of XIST expression is combined with differentiation potential of pluripotent stem cells (Martin et al. 1978; Dhara and Benvenisty 2004; Anguera et al. 2012).

Female hiPSCs with loss of XIST were reported to form poorly developed cystic teratomas, similar to the FES 30 teratomas in our study. The international Stem Cell Initiative characterized 59 hESC lines from 17 laboratories (Adewumi et al. 2007), including the Finnish FES 21, 22, 29 and 30 lines. In this study, all male lines expressed low or undetectable level of XIST but there was heterogeneity between female lines. In part of the XX lines, expression of XIST was very low or undetectable, while in the other part XIST was clearly detectable. FES 30 belonged to the former group and this can explain the results of differentiation in our study. However, it is not clear whether XIST expression is directly responsible for the differentiation behaviour.

Our results showed that gene expression profile and cell surface pluripotent markers assess hESC quality but they do not predict the differentiation capacity of pluripotent cells. The variation in the differentiation potential of hESC lines must be carefully considered by those comparing various differentiation methods. Later, it has been verified that differences in epigenetic adaptation as well as transcriptional variation are common among human ESC lines as well as in iPS cell lines, and this variation may have a significant impact on a cell line's utility (Allegrucci et al. 2007; Calvanese et al. 2008; Bock et al. 2011)

In the second part of this study we analyzed mRNA expression of early germ cell-specific and germ cell-enriched genes DAZL, STELLA, PUM2, PIWIL2, and TEX14 (Clark et al. 2004; Zwaka and Thomson 2005) in undifferentiated cells, EBs and outgrowth from EBs (stage 3 cells). Both undifferentiated stem cells and EBs expressed all markers but stage three cells were negative for all markers except TEX14 and PUM2, which showed weak expression (Table 11).

Table 11. Gene expression in undifferentiated stem cell lines, embryoid bodies (EBs) and plated EBs (Stage3).

	OCT4	NANOG	PUM2	DAZL	PIWIL	STELLAR	TEX14
FES lines	+	+	+	+	+	+	+
EBs	+	+	+	+	+	+	+
Stage 3	-	-	+	-	-	-	+

Our question was whether human ES cells might actually be more closely related to PGCs than epiblastic cells and we noticed that undifferentiated hESC express early germ cell-specific genes but it would be difficult to distinguish these two cell types. We suggested that early germ cell associated genes could be specific markers for hESC cells and that they may be involved in self-renewal or they might regulate molecular mechanisms of early differentiation.

5.3 PUBLICATION III: THE N-GLYCOME OF HUMAN EMBRYONIC STEM CELLS

The focus of study III was to characterize the N-glycan profile in undifferentiated hESC and its modifications during differentiation. Four hESC lines (FES 21, 22, 29 and 30) growing on human feeders were used for glycome analysis. Embryoid bodies and outgrowth from EBs (stage 3 cells) were used for comparison against undifferentiated hESCs. Both mouse and human feeder cells, culture media and its supplements were analysed to exclude potential contamination sources. This showed that the detected major glycan structures were not present in the potential contamination sources and N-glycan purity in the cell samples was estimated to be at least 93%.

5.3.1 N-glycans in hESC lines

In the analysed hESC samples (approximately 100 000cells /sample), the neutral N-glycans comprised about two third of the combined neutral and sialylated N-glycan pool. The mass spectrometric analysis revealed in each cell line the relative proportions of approximately 40 neutral glycan signals. In neutral N-glycan fraction the high-mannose N-glycans dominate the profile and five signals (H8N2, H9N2, H6N2, H7N2, and H5N2) comprised over 70% of the neutral N-glycan pool (Table 12). The profiles were similar, 15 of the 20 most abundant neutral N-glycan signals were the same in all lines.

The acidic N-glycan profiles of the four cell lines resembled each other even if there were more variation between cell lines than in the neutral N-glycan profiles. The complex-type N-glycans dominated the acidic N-glycan profile and all the major signals in this N-glycan pool contained sialic acid residues (S) (Table 12). The four most abundant acidic N-glycan signals contained the H5N4-core (Table 12) and they differed only by variable number of deoxyhexose (F) and sialic acid. The result showed that many of the sialylated N-glycans were multifucosylated, and comparing the acidic N-glycan profiles between undifferentiated and differentiated cells we found that complex fucosylation (F>1) was the most characteristic feature of undifferentiated hESCs.

Table 12. Fourteen most abundant mass spectrometric N-glycan signals of undifferentiated hESCs and embryoid bodies.

Neutral N-glycan			**Acidic N-glycan**		
Composition	% in hESC	% in EB	Composition	% in hESC	% in EB
H8N2 ***High-mannose***	19	13	S1H5N4F1 *Complex-type*	25	19
H9N2 ***High-mannose***	18	14	S1H5N4F2 *Complex-type*	11	2.9
H6N2 ***High-mannose***	16	16	S2H5N4F1 *Complex-type*	6.9	3.2
H7N2 ***High-mannose***	11	10	S1H5N4 *Complex-type*	5.0	3.6
H5N2 ***High-mannose***	8.8	12	S1H6N5F1 *Complex-type*	4.3	2.7
H5N4F1 ***Complex-type***	2.5	1.3	S1H4N5F1 *Complex-type*	2.6	3.4
H10N2 ***Glycosylated***	2.4	1.7	S1H6N5F2 *Complex-type*	2.5	0.7

H hexose, *N* N-acetylhexosamine, *F* deoxyhexose, *S* N-acetylneuraminic acid

The data demonstrated that hESCs prefer the production of both relatively unprocessed large high-mannose type N-glycans and fully prosessed biantennary complex-type structures. Similar findings were made from cord blood derived CD34+ and CD133+ hematopoietic stem cells (Hemmoranta et al. 2007) and bone marrow derived mesenchymal stem cells (Heiskanen et al. 2009). The high-mannose type N-glycans increase also upon induction of pluripotency in hiPSCs (Hasehira et al. 2012).

We found also N-glycans containing N-glycolylneuramic acid (G) in undifferentiated hESC samples. It has been shown that stem cells are able to absorb molecules like N-glycolylneuramic acid from their surroundings during culture, and incorporate them into their own cell surfaces (Martin et al. 2005; Heiskanen et al. 2007). However, its expression decreases upon culture in the absence of animal products (Heiskanen et al. 2007).

5.3.2 N-glycan profile changes during differentiation

In order to determine whether the N-glycome undergoes changes during differentiation, N-glycan profiles from undifferentiated hESCs, the embryoid bodies

and cells further differentiated from them (stage 3 cells) were compared. Discrimination analysis was used to distinguish distinct sets of findings and allocate new findings to undifferentiated and differentiated cells. The N-glycan profiles of the differentiated cell types differ significantly from the profile of undifferentiated hESC (Figure 13). There were many signals present in hESCs and EBs that disappeared in stage 3 cells. The acidic N-glycan profile was modified radically during differentiation. The five most abundant acidic N-glycan signals were different in all studied cell types. The major differentiation-associated changes were in their monosaccharide compositions. The most characteristic feature in undifferentiated hESC, complex fucosylation, was gradually decreased during differentiation. Fucosylation is involved in many cell signalling and adhesion events. Our previous gene expression study (Skottman et al. 2005) demonstrated that the α1,2-fucosyltransferase genes, FUT1 and FUT2, were overexpressed in undifferentiated hESCs comparing to EBs. This correlates well with the present finding that complex fucosylation takes place in N-linked glycans of hESCs. This suggests that glycan biosynthesis is controlled differently in hESCs, EBs and stage 3 cells. Overall, 29% and over 40 % of sialylated N-glycans were changed at EB stage and at stage 3, respectively, when compared to hESCs.

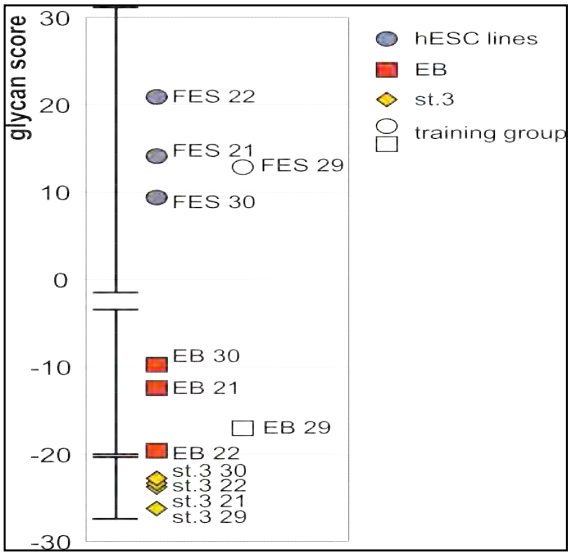

Figure 13. Identification of differentiation stage from the N-glycome data. To test whether the obtained N-glycan profiles could be used for reliable identification of hESC and differentiated cells even with the presence of sample-to-sample variation, a data analysis was performed. The FES29 and embryoid bodies derived from it (EB 29) were selected as the training group for the calculation. The resulting equation was applied to the other samples that served as the test group in the analysis and the results are described graphically. hESCs and the differentiated cell samples were clearly discriminated from each other by 2-tailed Student's t-test with Welch's approximation ($p < 0.01$). Furthermore, the stage 3 (st.3) differentiated cell samples were separated from the EB samples by 2-tailed Mann-Whitney U-test ($p<0.05$). The predicted 95% confidence intervals are shown for the three cell types.

The changes were not quite as marked among neutral N-glycans (14 % change at EB stage and 15 % at stage 3). In the neutral N-glycan pool, the high-mannose type N-glycans, which present immature biosynthesis products of N-glycans (Stanley et al. 2009), were common in all studied cell types. However, the EBs and stage 3 cells produced a wider variety of N-glycan types than hESCs, including low-mannose, hybrid-type, and monoantennary N-glycans even if the glycan profile did not change as much as the acidic N-glycans. Both hybrid and complex-type N-glycans have been suggested to have a role in development. Hybrid-type N-glycans may have special functions in proliferation (Schraen-Maschke and Zanetta 2003). Altogether, approximately one fourth of the N-glycan structures were changed when the hESCs differentiated into stage 3 cells. However, the biological consequences of the observed glycosylation changes during differentiation are still unknown.

5.3.3 Analysis of complex fucosylation

In addition to MS and NMR profiling, further structural information was needed for the assignment of glycan structures. Terminal epitopes, such as sialic or fucose residues bring structural diversity to N-glycans. The α1,6 –fucosylation is the most common fucosylation in human N-glycans (Staudacher et al. 1999) and it was found also in our N-glycan profile: the most common fucose residue in both hESCs and EBs was the N-glycan core α1,6-Fuc structure. However, in human N-glycans containing more than one fucose there should be other fucose linkages as well. Indeed, using exoglycosidase analysis we found that α1,2- and α1,3/4-fucosidase enzymes were characteristic to complex fucosylation in hESCs. The galactosidase assay results suggested that the fucosylated antennae could be either α1,2-fucosylated H type 2 or α1,3-fucosylated Lewis X (Le^x).

5.3.4 Validation of the N-glycomic analysis

Since the mass spectrometric and NMR spectometric profiling of hESC glycome comprised the overall cellular glycome, including intracellular glycans, further verification of cell surface presentation of the observed glycan structures was needed. Lectin proteins recognize glycans with specificity to certain glycan structures so we used lectins to validate our N-glycan profile data on the cell surface We stained hESCs using three lectins: *Ulex europaeus* agglutinin I (UEA I) that recognizes especially H type 2 (Fucα1-2Galβ1-4GlcNAc), and *Maackia amurensis*

agglutinin (MAA) recognizing α2,3-linked sialic acid containing structures, specifically Neu5Acα2-3Galβ1-4GlcNAc. To recognize α-mannosylated structures and potentially core fucosylated N-glycans, we used *Pisum sativum* agglutinin (PSA). The specificity of lectin staining was validated with specific glycan inhibitors (Figure 12). The experiments thus demonstrated that both complex fucosylated structures α2,3-sialylated LacNac and H-type 2 were abundant on the surface of undifferentiated hESCs. The cell surface of hESCs was not labeled by PSA (Figure 12) so α-mannosylated structures were not expressed on cell surface.

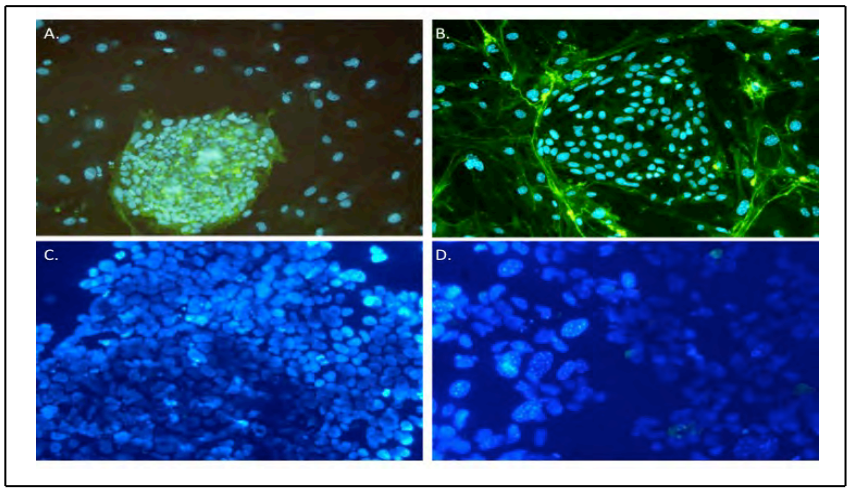

Figure 12. To study localization of glycan components, hESC were grown on mouse feeder cells and labeled by fluorescent glycan-specific reagents selected based on analysis results. A. The hESC surfaces were stained by Maackia amurensis agglutinin (MAA) indicating that α2,3-sialylated glycans are abundant on hESC but not on MEF. B. In contrast, the hESC surfaces were not stained by Pisum sativum agglutinin (PSA) that recognized α-mannosylated glycans on MEF. Addition of competitive inhibitors blocks lectin binding. C. 3'-sialyllactose blocks MAA binding, and D-mannose blocks PSA binding (D).

Although the biological significance of this study remained elusive, it generated a global picture of the N-glycans occurring in hESC glycoproteins. Taken together, the data indicated that there was only slight variation between cell lines, overall they shared about 85% of the total detected N-glycans. We also suggested that the N-glycan profile of hESC consists of two parts, constant and variable. In the constant part, the major glycan types were high-mannose and biantennary complex-type N-glycans and in the variable part the most characteristic feature was complex fucosylation. Changes in the monsaccharide compositions of the expressed N-glycans suggest that glycan biosynthesis is controlled differently in hESCs and at the differentiation stage.

Pluripotency-associated antigens on the cell surface are often glycoproteins or

glycolipids (Kannagi, et al. 1983a,b; Andrews et al. 1984a). Beyond those antibodies and lectins now widely used in monitoring the stem cell status, there is a need for discovering new markers as well as for further characterization of the function of the existing markers. This study highlights the importance of protein glycosylation in hESCs and shows that they have a unique N-glycan profile, which is modified during differentiation. Recent data (Toyoda et al. 2011; Wang et al. 2011) together with this study support the idea that post-translational glycosylation of cell surface proteins might play a role in the regulation of transitions between pluripotent and differentiated states that occur during differentiation of pluripotent cells as well as reprogramming of somatic cells. However, further analyses of the global protein glycosylation status in a broad spectrum of hESCs and hiPSCs and their differentiated derivatives are still needed.

5.4 PUBLICATION IV: LONG-TERM SELF-RENEWAL OF HUMAN PLURIPOTENT STEM CELL ON *ERYTHRINA CRISTAGALLI* LECTIN

Cell surface glycans contribute to the adhesion capacity of cells and are essential for cellular signal transduction. In publication III (Satomaa et al. 2009) we studied glycan epitopes on the stem cell surface and in this study (Publication IV) we tested the capacity of these glycans to support self-renewal of pluripotent stem cells in long term culture.

Based on the previously identified glycans typical for undifferentiated hESCs (paper III), we tested a number of lectins specific for these epitopes to support the long-term culture of undifferentiated ESCs. Four lectins ECA (*Erythrina Cristagalli* agglutinin, binding specificity to type 2 N-acetyl-lactosamine structures), MAA (*Maackia amurensis* agglutinin, specific for α2,3-linked sialic acid*)*, WFA (*Wisteria floribunda* agglutinin, binding preferentially to N-acetylgalactosamine in α- or β-linkage) and PWA (*Phytolacca americana* agglutinin, with N-acetylglucosamine specificity, binding also to polylactosamine structures) were tested for their ability to act as a growth supporting matrix for hESC lines. Cells were attached only onto *ECA* and *MAA* but cells differentiated on MAA. Since the ECA lectin emerged as the most promising substrate, we then cultured one hESC line (FES 29) and one hiPSC line (HEL11.4) for over 20 passages (over three months) on ECA-coated cell culture dishes in mEF conditioned media, and also at least 10 passages in defined medium (StemPro, Life Technologies). The cells were passaged in small aggregates every 3-4 days at a 1:3-1:5 ratio with collaganese IV and cultured in medium supplemented

with Pinacidil (Barbaric et al. 2010; Barbaric et al. 2011). The results showed that the hPSCs maintained pluripotency and proliferation capacity after prolonged culture equally well on ECA as on Matrigel™ (Figure 14).

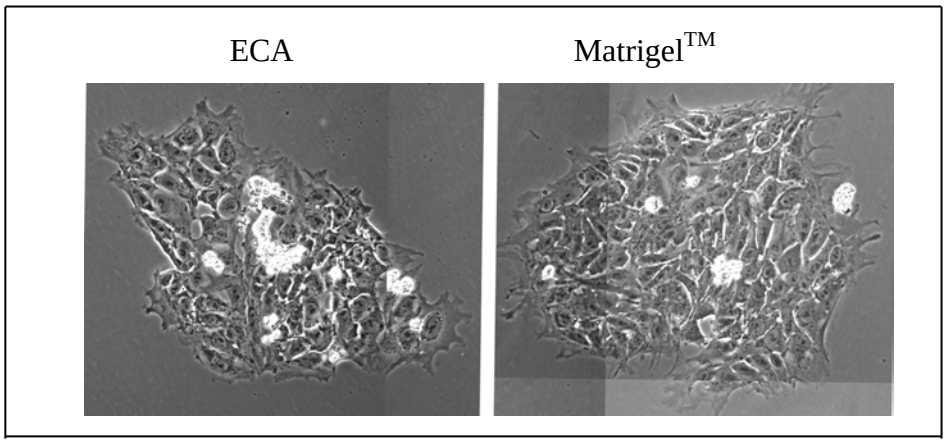

Figure 14. Pluripotent stem cell (FES 29) morphology on ECA and Matrigel™. The images are captured in a live cell imaging system Cell IQ (CM-Technologies). Objective magnification 10x.

Cells on ECA maintained the pluripotency markers OCT4, SOX2, NANOG and E-cadherin as revealed by immunostaining. FACS analyses of cell surface markers TRA-1-60, SSEA-3, H-type 1 and SSEA-1 expression were performed on ECA and Matrigel™ every fifth passage. No significant differences were detected in the stem cell marker expression of hESCs cultured either on ECA or on Matrigel™ (Figure 15).

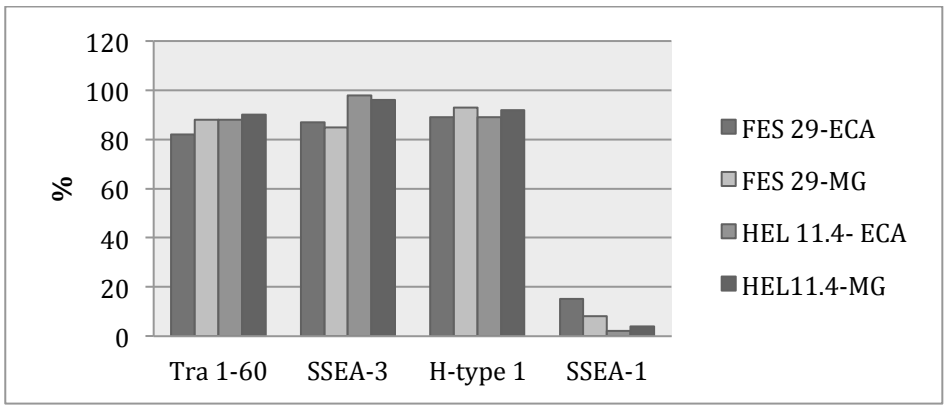

Figure 15. FACS analysis of pluripotency-associated cell surface markers after 20 passages on ECA. No differencies between cells growing on ECA or Matrigel™ were detected.

The expression of the main pluripotency-associated genes was also demonstrated by quantitative-PCR. The expression of pluripotency genes OCT4, SOX2 and NANOG and primitive streak/early differentiation markers Brachyury and Goosecoid were analysed throughout 20 passages on ECA and

Matrigel™. The expression levels of pluripotency genes remained similar; no significant differences were detected, some minor upregulation of Brachyury and Goosecoid occurred at later passages of FES29 cells on ECA. With the HEL11.4 cell line, the pluripotency genes tended to remain higher and the differentiation genes lower on ECA throughout the culture period. In addition, at passage 9 a bigger panel of key genes involved in the maintenance of pluripotency and the self-renewal status of embryonic stem cells was performed using PCR-arrays (SABiosciences). We studied also the gene expression profile of molecules involved in cell-to-cell and cell-to-matrix interactions. The mRNA expression profiles of about 80 genes related to pluripotency and early differentiation or ECM molecules showed no significant differences (over 2-fold) between cells cultured on ECA *vs.* Matrigel™(Figure 16 A). Surprisingly, we did not see any difference (over 2-fold) between ECA and Matrigel™ in adhesion assay (Figure 16 B).

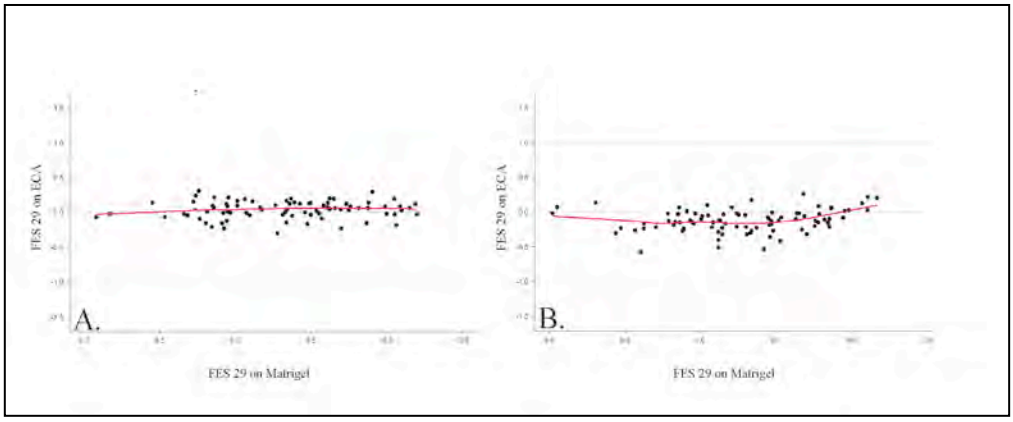

Figure 16. Gene profiles were compared between FES 29 culture 9 passages on Matrigel™ and on ECA. A. Embryonic stem cell array determined 84 key genes involved in the maintenance of pluripotency and the self-renewal status of embryonic stem cells. B. The Extracellular Matrix and Cell Adhesion PCR Arrays determined the 84 gene expression profile of molecules involved in cell-to-cell and cell-to-matrix interactions. Y-axis (log_2) is the intensity ratio and X-axis is the average intensity for a given gene measured on two similar HTqPCR arrays. All differences were less than two-fold.

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The pluripotent cells were seeded sparsely (35 cells/cm^2) from ECA and Matrigel™ onto both ECA and Matrigel™ coated plates. Colonies were fixed with 4% paraformaldehyde, stained with alkaline phosphatase-solution and counted (Figure 17). Significant differencies were detected between matrixes. More than 10% of cells plated from ECA onto ECA formed colonies as counted

after 10 days whereas only around 6 % of cells from Matrigel™ onto Matrigel™ or ECA formed colonies ($p<0.01$, one way ANOVA).

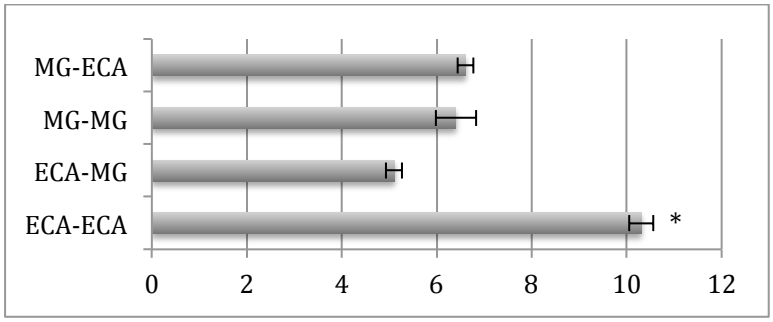

Figure 17. Clonogenic capacity on ECA and Matrigel™. Significantly better clonogenicity on ECA ($p<0.05$, oneway ANOVA).

The hPSC lines were cultured on ECA and Matrigel™ and monitored using a time-lapse imaging system Cell-IQ® (CM-Technologies, Finland) to study colony size area. Each region of interest was monitored every 60 minutes during four days. Analysis of areas occupied by the colonies as well as cell number in the colonies showed no significant differences (Figure 18).

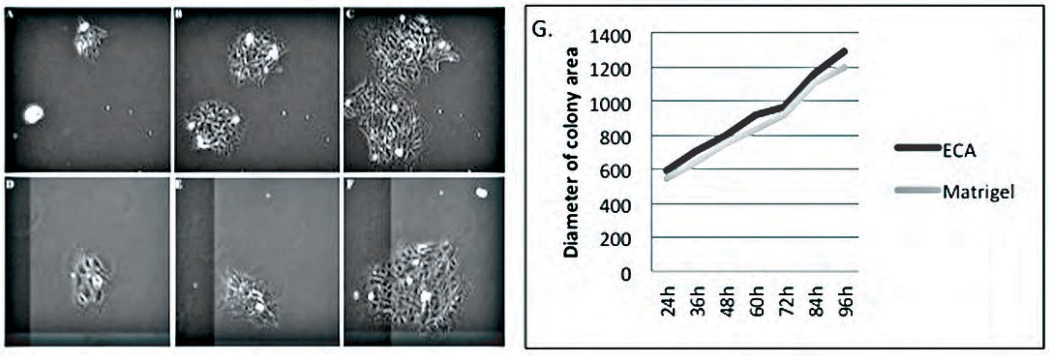

Figure 18. FES 29 cells growing on ECA (Day1 (A), Day 2 (B), Day 4 (C)) and Matrigel™ (Day1 (D), Day 2 (E), Day 4 (F). Cells were passaged in small clumps and cultured in live cell imaging system (Cell IQ, CM-technologies. Objective magnification 10x. .G. Areas of the colonies were analyzed in Cell IQ Analyzer program and results are shown as average size. Y-axis is the diameter of colony area counted as pixels. No differencies in colony size were detected.

The differentiation potential of cells growing on ECA was evaluated *in vitro* using the EB-differentiation assay and *in vivo* by the teratoma formation analysis. The EBs expressed markers from the three embryonic lineages; endoderm, ectoderm, and mesoderm, and structures from all these germ layers were also detected in the teratomas (Figure 19).

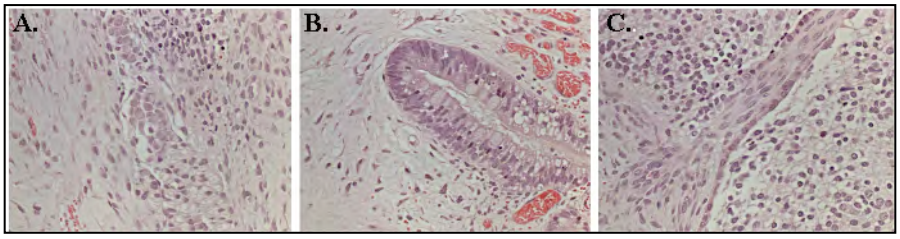

Figure 19. Teratoma from FES 29 growing on ECA. Cells from ectoderm (A), endoderm (B) and mesoderm (C) origin. Objective magnification 40x.

We validated the cell-ECA binding specificity with specific inhibitors. The results showed that lactose monohydrate ($LacH_2O$), composed of galactose β1,4-linked to glucose, and lactone-N-neotetraose (LnNT) composed of β1,4-linked galactose inhibited cell attachment effectively ($p<0.001$, between inhibitors and control) (Figure 20). We also used saccharide control, saccharose that is composed of fructose α1,1-linked to glucose. It had no inhibitory activity ($p>0.6$). This indicated that initial cell attachment to the growth surface was dependent on a specific interaction of the surface-bound lectin with stem cell glycan ligands.

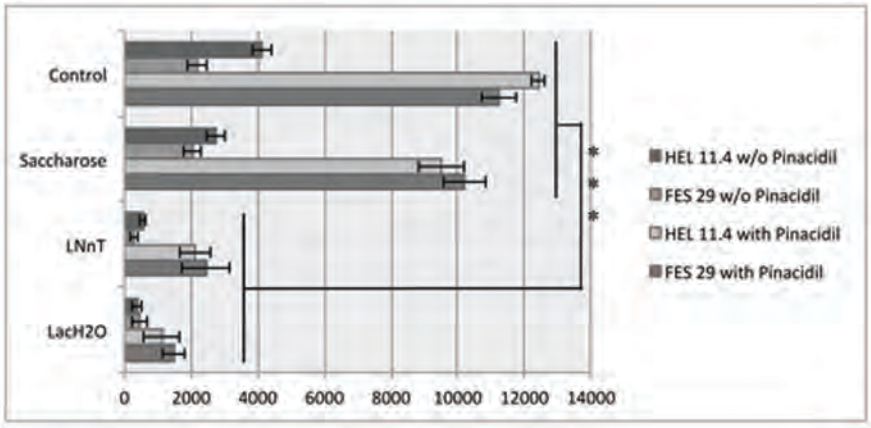

*Figure 20. Validation of the binding specificity. Specific saccharide inhibitors lactose monohydrate ($LacH_2O$) and lacto-N-neotetraose (LnNT) inhibited significantly cell attachment onto ECA (***,p<.001) . The control saccharide, saccharose, had no inhibitory activity*

In addition, we evaluated the *in vitro* differentiation potential of FES 29 and HEL11.4 cells to definitive endoderm and hepatic precursor cells on ECA. Definitive endoderm (DE) differentiation was verified by the gene and protein expression of the endoderm markers FoxA2 and Sox17, and the anterior DE marker Cerberus (CER1) as well as FACS analysis for the cell surface marker CXCR4. On top of Matrigel^{TM} the cells formed a confluent single cell layer of typical DE cells. However, cells differentiated on ECA were found to detach easily from plates during DE induction leading to the loss of a significant proportion of cells (Figure 21).

However, the cells remaining on the plate showed normal DE differentiation (Figure 21).

After five days of DMSO treatment at stage 2, the cells formed hepatic endoderm with alfafetoprotein (AFP) positive hepatic progenitors. During the later maturation stage, albumin expression increased rapidly and the cells obtained morphology typical of hepatocytes. However, also AFP expression continued to rise, which demonstrates the still immature nature of the hepatocyte-like cells. Overall, there were no qualitative differences between cells grown on ECA or Matrigel™.

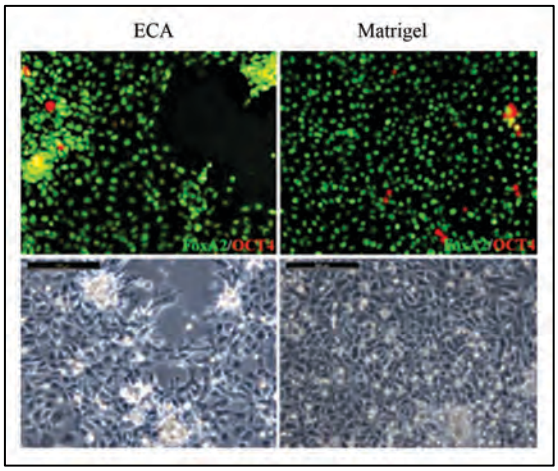

Figure 21. Immunocytochemistry (FoxA2 green, OCT4 red) and morphology pictures of definitive endoderm cells. Detachment of cells from ECA matrix is visible.

ECA is a fully defined cell culture matrix offering several advantages for the standardization of human pluripotent stem cell cultures. We demonstrated that ECA supports self-renewal and pluripotency both in serum-containing and in defined serum-free media. The expression of pluripotency markers and functional characteristics of cells cultured on ECA were comparable to cells on Matrigel™. However, binding of the stem cells to ECA is relatively weak, which may cause problems at the time of passaging or during the induction of differentiation. We found that the use of Pinacidil (or ROCK inhibitor) at the time of passaging is obligatory to maintain a sufficient number of viable cells for efficient expansion. With the combination of ECA plus Pinacidil, the attachment and viability of dispersed single stem cells was better than on Matrigel™ plus Pinacidil. This resulted in higher clonogenic efficiency on ECA, which makes it a promising and cost-effective tool for the study of human pluripotent stem cell biology.

6. CONCLUDING REMARKS

Since the isolation of the first hESC lines, a lot of effort has been put into understanding the molecular factors and signalling pathways in self-renewal and controlled differentiation of pluripotent cells. Traditional characterization of pluripotent stem cells means analysis of morphology, marker expression and developmental capacity. However, better tools would be needed to predict the differentiation propensity of the cell lines without time-consuming and expensive experimentation. Such tools may be developed on the basis of global genetic and epigenetic analyses. The deviation scorecard (Bock, Kiskinis et al. 2011) is an example of this approach, promising a strategy for the prediction of lineage differentiation propensity already at the undifferentiated state. In addition to genomics and proteomics, the complex cell surface glycan structure of the cell membrane may provide useful keys for the characterization of the hPSCs.

The purpose of this thesis was to investigate culture conditions for hPSC lines and study characteristics of pluripotent stem cells and their differentiated progenies based on their glycan profiles. These studies provided new insights into culturing and characterization of stem cells. The main conclusions are:

- Derivation and culture of hESC lines on human foreskin fibroblasts instead of foetal mouse fibroblasts represents an improvement in culture conditions towards clinical quality.

- The derivation and characterization of the first Finnish embryonic stem cell lines demonstrates that human embryonic stem cells share many markers with primordial germ cells and these markers may be used to identify pluripotent stem cells. Furthermore, the results show that hESC lines differ in their capacity to differentiate and better tools are needed to predict cell lineage propensity.

- Global N-glycan profiling of hESCs and their differentiated derivatives demonstrates specific glycan fingerprints of pluripotent stem cells and their early differentiation derivatives. Furthermore, the specific binding properties of various lectins can be utilized to reveal the glycan determinants on the cell surface. These results emphasize the importance of protein glycosylation in hPSCs and shows that systematic glycan biology analysis of stem cells may provide valuable tools for the analysis of

pluripotent stem cells.

- A platform for stem cell culture was developed using *Erythrina Cristagalli (ECA)* lectin. This method enabled high clonogenic efficiency and effective expansion of both hESC and hiPSC lines while maintaining characteristic stem cell marker expression and pluripotency comparable to the commonly used matrix, Matrigel^{TM} ECA lectin is proposed as a cost-effective defined matrix for various studies of human pluripotent stem cells.

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