Expression of Tissue Antigens in Human Pluripotent Stem Cells and Alterations During Differentiation

Potential application in regenerative medicine for treatment of terminal cell and organ failure

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Gothenburg 2017

Cover illustration: Electron microscopy image of human pluripotent stem cells stained with an antibody against sialyl-lactotetra.

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ISBN 978-91-629-0217-9
ISBN 978-91-629-0218-6 (PDF), http://hdl.handle.net/2077/51885

Printed in Gothenburg, Sweden 2017
Ineko AB

To my family,

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ABSTRACT

The major limiting factor in the treatment of patients with end-stage organ failure is the insufficient number of human organs available for transplantation. An unlimited access to human cells, tissues and organs would also open up possibilities to treat several chronic diseases, such as diabetes, neurological and cardiovascular diseases, affecting millions of patients worldwide. Cells and tissues derived from human pluripotent stem cells (hPSC) could potentially fulfill these ambitions. However, there are several biomedical barriers to overcome before this can be a clinical reality. One of the most important concerns is the immunogenicity of the conceivable cell or tissue grafts derived from hPSC when exposed to a non-self recipient.

This thesis explores the expression of immunogenic tissue HLA and blood group antigens in several hPSC cell lines and their derivatives. This characterization was performed by several complementary analytical techniques, such as flow cytometry, immunohistochemistry, PCR, as well as biochemical characterization of glycosphingolipid molecular structures and protein bound antigen composition. The results demonstrate that pluripotent stem cells express various cell surface immunodeterminants including HLA, AB(O)H and related histo-blood group antigens. Moreover, we identified significant alterations of antigen expression patterns during endodermal, mesodermal and ectodermal differentiation. Consequently, our results indicate that all hPSC-derived cells intended for clinical applications should be characterized regarding their individual tissue antigen profile in accordance with the standard selection criteria used in allotransplantation. Furthermore, we identified a novel cell surface marker of undifferentiated stem cells, sialyl-lactotetra, which can be used as a verification and selection tool for pluripotency, as well as a potential exclusion measure in heterogeneously differentiated cell cultures to prevent tumor formation.

In conclusion, this thesis adds new knowledge regarding cell surface antigen expression in hPSC of relevance both for basic science and for future clinical applications within transplantation and regenerative medicine.

Keywords: Pluripotent stem cells, Differentiation, Histo-blood group antigens, HLA, Tissue antigens, Cell surface antigens, Sialyl-lactotetra, Transplantation, Regenerative medicine.
ISBN: 978-91-629-0217-9 (Print), 978-91-629-0218-6 (PDF), http://hdl.handle.net/2077/51885

SAMMANFATTNING PÅ SVENSKA

Bristen på humana celler, vävnader och organ tillgängliga för transplantation är den viktigaste begränsande faktorn för behandling av patienter som lider av sviktande eller upphävd funktion i såväl hela organ, som hjärta, njure eller lever, som i celler med specialiserad funktion vilket resulterar i sjukdomar som t.ex. diabetes, Parkinsons sjukdom eller åldersförändringar i gula fläcken. De senaste decenniernas medicinska framsteg har resulterat i nya möjligheter att odla fram omogna celler, så kallade pluripotenta stamceller, från människa. Dessa celler kan i laboratoriemiljö stimuleras till att bilda de flesta av kroppens olika celltyper. Därmed utgör de en potentiellt obegränsad källa till celler, vävnader och organ för behandling av en rad olika sjukdomar. Det kvarstår dock ett omfattande vetenskapligt arbete för att detta ska kunna bli en klinisk realitet. För närvarande pågår ett antal kliniska studier på patienter där humana pluripotenta stamceller ska ersätta skadade celler och vävnader. En förutsättning för att dessa transplantat ska fungera, och därmed uppnå sitt terapeutiska syfte, är att de inte framkallar en avstötningsreaktion hos mottagaren. De viktigaste faktorerna att beakta i detta avseende är specifika molekyler på cellernas yta, s.k. vävnadsantigen, vilka kan interagera med de vita blodkropparna i mottagarens immunsystem.

I denna avhandling har vi noggrant kartlagt uttrycket av ett flertal vävnadsantigen i ett antal humana pluripotenta stamcellslinjer samt i nerv-, hjärt- och lever-liknande celler som härstammar från dessa omogna stamcellslinjer. Analyserna genomfördes med hjälp av etablerade laboratoriemetoder som genotypning, immunidentifiering samt biokemiska studier av cellernas protein- och lipidsammansättning.

Vi identifierade en tidigare okänd markör för pluripotenta stamceller vid namn sialyl-lactotetra, som kan användas för att verifiera att en cellkultur består av omogna celler och för att särskilja omogna celler från mer utmognade celler. Vidare har vi påvisat att de pluripotenta stamcellerna uttrycker vävnadsantigen på sin cellyta, såsom HLA- och ABO-blodgruppssystemet, vilka kan stimulera immunsystemet hos en mottagare. Följaktligen måste man ta hänsyn till dessa vävnadsantigen när de pluripotenta stamcellerna, eller vävnader skapade från dessa celler, transplanteras i terapeutiskt syfte. Dessutom förändras uttrycket av vävnadsantigen under cellmognadsprocessen enligt ett till synes individuellt mönster och uttrycket kan således inte generaliseras, utan måste karakteriseras för varje enskild cellinje innan klinisk tillämpning.

Sammanfattningsvis har denna avhandling tillfört ny kunskap om uttrycket av vävnadsantigen hos omogna stamceller samt mogna celltyper som har sitt ursprung från dessa celler. Denna nya kunskap är betydelsefull och kan tillämpas inom såväl grundvetenskaplig medicinsk forskning som vid framtida kliniska behandlingar av patienter som lider av sjukdomar med sviktande funktion i kroppens celler eller organ.

LIST OF PAPERS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I. Barone A, **Säljö K**, Benktander J, Blomqvist M, Månsson JE, Johansson BR, Mölne J, Aspegren A, Björquist P, Breimer ME, Teneberg S.

Sialyl-lactotetra, a novel cell surface marker of undifferentiated human pluripotent stem cells.

Journal of Biological Chemistry 2014; 289, 18846-18859.

II. **Säljö K**, Barone B, Vizlin-Hodzic D, Johansson BR, Breimer ME, Funa K, Teneberg S.

Comparison of the glycosphingolipids of human-induced pluripotent stem cells and human embryonic stem cells.

Glycobiology 2017; 27: 291-305.

III. **Säljö K**, Barone A, Mölne J, Rydberg L, Teneberg S, Breimer ME.

HLA and Histo-Blood Group Antigen Expression in Human Pluripotent Stem Cells and their Derivatives.

Manuscript, submitted.

CONTENT

ABBREVIATIONS

PSC	Pluripotent stem cells	IC	Immunocytochemistry
hPSC	Human pluripotent stem cells	IF	Immunofluorescence
hESC	Human embryonic stem cells	CBA	Chromatogram binding assay
hiPSC	Human induced pluripotent stem cells	TLC	Thin-layer chromatography
EC	Embryonal carcinoma cells	EM	Electron microscopy
NSC	Neural stem cells	MS	Mass spectrometry
HLA	Human leukocyte antigens	SSEA	Stage-specific embryonic antigen
MHC	Major histocompatibility complex	S-Lc_4	Sialyl-lactotetra
Le^a	Lewis a	Lc_4	Lactotetra
Le^b	Lewis b		
Le^x	Lewis x		
Le^y	Lewis y		
S-Le^x	Sialyl-Lewis x		
FC	Flow cytometry		
IH	Immunohistochemistry		

Chemical structures and shorthand designations

Trivial name	**Antigen determinant**
H type 1/SSEA5	Fucα2Galβ3GlcNAcβ3-R
Globopenta/SSEA-3	Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Globoside/P-antigen	GalNAcβ3Galα4Galβ4Glcβ1Cer
Globo H	Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Sialyl-globopenta/ SSEA-4/Luke antigen	NeuAcα3Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Le^a	Galβ3(Fucα4)GlcNAcβ3-R
Le^b	Fucα2Galβ3(Fucα4)GlcNAcβ-R
Le^x/SSEA-1	Galβ4(Fucα3)GlcNAcβ-R
Sialyl-Le^x	NeuAcα3Galβ4(Fucα3)GlcNAcβ-R
Le^y	Fucα2Galβ4(Fucα3)GlcNAcβ-R
Blood group A antigen	GalNAcα3(Fucα2)Galβ-R
Blood group B antigen	Galα3(Fucα2)Galβ-R
Sialyl-lactotetra	NeuAcα3Galβ3GlcNAcβ-R
Sialyl-neolactotetra	NeuAcα3Galβ4GlcNAcβ-R
Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer
GD1a	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glcβ1Cer
GD1b	Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4Glcβ1Cer
GM3	Neu5Acα3Galβ4Glcβ1Cer
Sialyl-globotetra	Neu5Acα3GalNAcβ3Galα4Galβ4Glcβ1Cer

Trivial name	**Antigen determinant**
Sulf-globopenta	SO_3-3Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer

Key to glycan symbols

The symbolic presentation follows the recommendation outlined in "Essentials of Glycobiology" 2nd edition (1).


Galactose (Gal)


Glucose (Glc)


Mannose (Man)


N-Acetylgalactosamine (GalNAc)


N-Acetylglucosamine (GlcNAc)


N-Acetylneuraminic acid (Neu5Ac)


Fucose (Fuc)

1 INTRODUCTION

There is currently a globally increasing incidence and prevalence of patients suffering from chronic diseases that result in tissue and organ failure (2). Conditions such as ischemic heart disease, stroke, diabetes mellitus and chronic obstructive pulmonary disease are leading causes of morbidity and mortality worldwide.

Lack of function in, or extensive damage to, a tissue or organ leads to disease and ultimately even death. Transplantation of new organs from living or deceased donors is one therapeutic alternative available for patients in the industrialized countries. However, there is a critical shortage of donor organs. In the Scandinavian countries about 2300 patients are enlisted for transplantation annually. In Sweden alone, more than 800 patients are currently waiting for organs according to the Scandiatransplant organization (3).

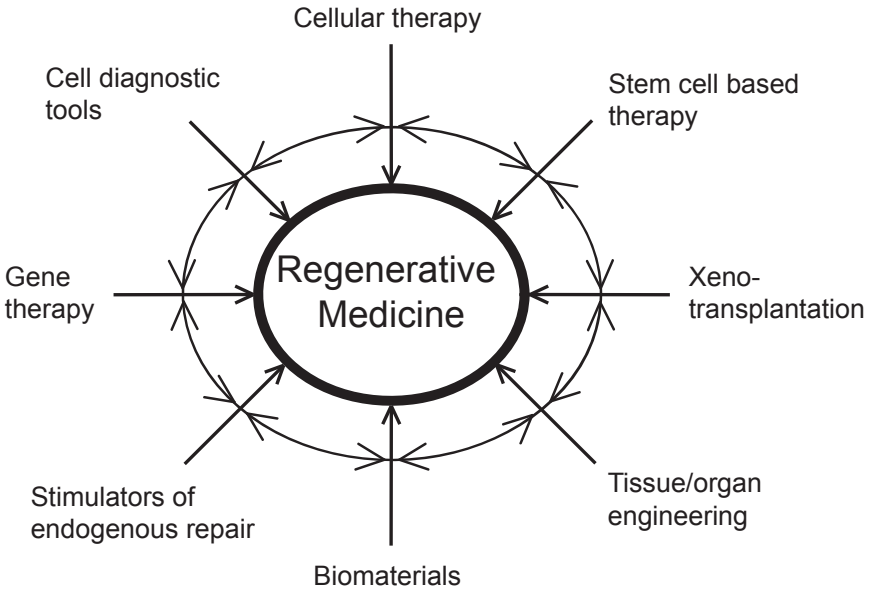

Figure 1. Illustration of the translational field of regenerative medicine*. The outer circle with arrows represents the tight associations and sometimes overlapping research areas.*

Instead of replacing organs, it would be desirable to repair and restore normal function by regeneration and replacement of cells and tissues. These alternative measures are the focus of regenerative medicine, an emerging field of research translating multidisciplinary biology, surgery and engineering science into therapeutic strategies. A wide variety of research fields and techniques are included in the term regenerative medicine, such as gene targeting, xenotransplantation, tissue engineering and stem cells research (schematically illustrated in Figure 1). The latter is the focus of this thesis, i.e. stem cell based therapies for treating end-stage tissue and organ failure.

1.1 Pluripotent stem cells

The successful isolation of embryonic stem cells (Figure 2) by Evans et al. (4) and concurrently by Martin et al. (5) from the inner cell mass of the blastocyst in mouse, and subsequently in human by Thomson et al. (6), marked the beginning of a new era of regenerative medicine. The field broadened even further with the derivation of induced pluripotent stem cells from adult mouse and human cells by Takahashi and Yamanaka et al. (7, 8). The contributions by Sir Martin J. Evans and Shinaya Yamanaka was rewarded the Nobel Prize in Physiology and Medicine in 2007 and 2012, respectively.

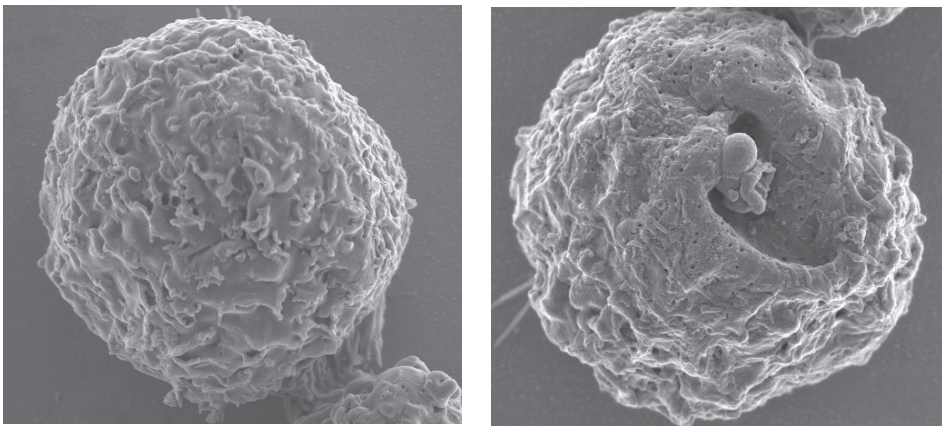

Figure 2. Scanning electron microscopy images of the cell surface of a pluripotent stem cell *(human embryonic stem cell line SA181).*

In the early days of stem cell research, pluripotent cells were cultivated on so called feeder cells, usually mouse embryonic feeder cells, to promote and facilitate culture survival and expansion. Consequently, potentiating incorporation of animal components in human cells and enhancement of the immunogenicity of the transplant (9). This substantial hurdle has in recent years been overcome. Methodological, technical and media refinements and developments now enable culturing of pluripotent stem cells and their derivatives under feeder-free and even xeno-free conditions (10-12). These advancements have facilitated the transition from the lab bench to the clinic, resulting in numerous GMP (Good Manufacturing Practice) and clinical-grade PSC lines worldwide allowing several ongoing clinical trials (13).

The pluripotent stem cells (PSC) have unlimited ability to propagate *in vitro* (14) and differentiate into all three embryonic germ layers, i.e. endoderm, mesoderm and ectoderm. Figure 3 shows a schematic illustration of the formation of PSC and their differentiation into hepatocyte-like cells (endoderm), cardiomyocyte-like cells (mesoderm) or neural stem cells (ectoderm). All human cell types can thus be derived from human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), providing an infinite source of cells, tissues and possibly even organs with great therapeutic potential (15, 16), which can be used for tissue engineering and transplantation therapies. Derivatives from hPSC can also be used to treat numerous chronic diseases and lack of tissue due to malformations or trauma, e.g. skin after burn injuries. Consequently, the applications are many in the field of reconstructive surgery with the possibilities to provide tissues such as cartilage, skin and bone.

Furthermore, hPSC and their derivatives can be used in the pharmaceutical industry for identification of novel molecular targets for therapy development and toxicology screening, providing endless material for drug testing in cell-based disease and disease-free models. Moreover, since most drugs are metabolized in the liver, generation of healthy as well as diseased hepatocytes from hPSC entails more efficient *in vitro* testing and consequently enhancing toxicology and pharmacokinetic studies, potentially lowering the costs for drug development.

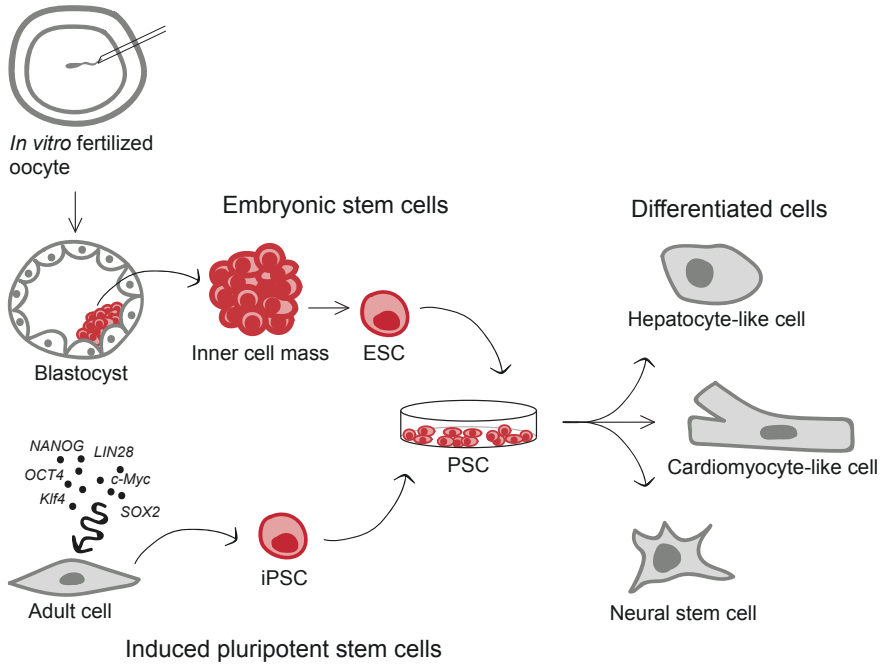

Figure 3. Schematic presentation of the formation of pluripotent stem cells (PSC). *Embryonic stem cells (ESC) isolated from the inner cell mass (red) of the blastocyst originating from an in vitro fertilized oocyte and induced pluripotent stem cells (iPSC) derived through reprogramming of adult cells. Following establishment, the PSC can subsequently be differentiated into the three embryonic germ layers, represented in this thesis by hepatocyte-like, cardiomyocyte-like and neural stem cells.*

Several studies have shown successful PSC-based replacement therapy for a wide range of diseases and end-stage organ failures in various animal models. For instance, hPSC-derived cells have been shown to survive, mature and function as midbrain dopamine-secreting neurons in rat models of Parkinson´s disease (17, 18). Transplanted mouse iPSC-derived neural stem cells have been shown to successfully differentiate *in vivo* into all three neural lineages and promote locomotor function recovery in spinal cord injured mice (19). Furthermore, gene-targeted mouse iPSC differentiated into hematopoietic progenitors have been shown to cure humanized sickle cell anemia after autologous transplantation (20). Moreover, hPSC-derived pancreatic β-cells have been demonstrated to secrete human insulin into the serum of mice in a glucose-regulated manner (21), reversing the progressive hyperglycemia normally observed in the validated diabetic mouse model used.

Human pluripotent stem cells and their derivatives are successfully cultured *in vitro*. However, hPSC were initially only cultured in monolayers, which is not adequate for tissue or organ replacement therapies. In recent years a lot of efforts have therefore been made within regenerative medicine and the field of tissue engineering in particular to develop new strategies to enable production of 3D formations. In a proof-of-concept study of self-organizing 3D structures by Takebe et al., hiPSC-derived liver buds were generated (22). Subsequent transplantation into immunodeficient mice induced vascularization and differentiation into functional liver cells producing albumin and human-specific drug metabolism of e.g. ketoprofen. Furthermore, the hiPSC-derived liver buds improved survival after drug-induced liver failure in a validated mouse model.

Currently, there are several on-going clinical phase I and II studies using hPSC-derived cells and tissues, including age-related macula degeneration, Parkinson´s disease, spinal cord injury, diabetes mellitus and myocardial infarction (reviewed in (13, 23)).
Despite these astonishing possibilities, there are many aspects that need to be addressed before PSC-derived products can be used in the clinic, such as the *in vivo* functionality, risk for tumorigenic potential and immunological rejection of the graft.

Pluripotency characteristics

The term and property of pluripotency has been known since the late 19th century and its historical development is thoroughly reviewed by Robinton and Daley (24), from the first encounter working with blastocysts from sea urchin by Driesch in 1891 (25) to the isolation of hESC by Thomson et al. (6) more than a hundred years later. Preceding the isolation of mouse embryonic stem cells in 1981 (4, 5), pluripotency and early embryogenesis were mainly studied in embryonal carcinoma cells (EC), i.e. stem cells originating form teratocarcinomas. Embryonal carcinoma cells are considered the malignant counterpart of embryonal stem cells (26).

Different assays for evaluating pluripotency have been utilized, including genomic profiling of pluripotency genes, phenotypic profiling using immunostaining methods with established pluripotency markers and functional assays assessing the ability of embryoid body or teratoma formation (27). The golden standard for functional validation of pluripotency is the cells' capability of *in vivo* teratoma formation, i.e. forming well-differentiated tumors containing elements form all three embryonic germ layers when injected (subcutaneously or intramuscularly) into immunodeficient mice (28). Furthermore, spontaneous *in vitro*

differentiation into all three embryonic germ layers is a functional assay of true pluripotency.

Markers of pluripotency

The term pluripotency genes refers to the genes expressed in PSC encoding the pluripotency transcription factors known to regulate a cell's ability to differentiate into all three embryonic germ layers. The most established pluripotency transcription factors are OCT4 (octamer-bindning transcription factor 4), SOX2 (sex determining region Y-box 2) and homeobox protein NANOG, because of their capability to activate downstream targets that regulate self-renewal, differentiation processes, as well as their own genes (29, 30), hence promoting pluripotency through an interconnected autoregulatory circuitry and positive feedback loop. Other transcription factor proteins such as REX-1 are also widely used as intracellular pluripotency markers. Furthermore, high expression of alkaline phosphatase is associated with pluripotency and has been widely used as a marker for undifferentiated stem cells (31, 32), with some exceptions such as expression in insufficiently reprogrammed hiPSC cells (33) and during differentiation of mesenchymal stem cells (34).

A lot of focus and efforts have been made to establish specific markers of pluripotency, especially markers accessible for analysis with different immunostaining methods, thereby facilitating identification and selection of pluripotent cells from a heterogeneous population or lineage-differentiated cells as well as analysis of differentiation. Results from several studies have shown the necessity to analyze multiple markers simultaneously (33).

A number of different cell surface antigens have been used as pluripotency markers to identify and select PSC. An extensive characterization was made by The International Stem Cell Initiative, who investigated 59 hESC lines worldwide, comparing genotypes and phenotypes including expression of a wide range of pluripotency markers (35). Commonly, the antibodies used in various immunostaining methods so far are directed mainly against carbohydrate epitopes (Figure 4). The expression-patterns of the pluripotency markers studied are similar in hESC and hiPSC in the naive state (36), as well as during differentiation.

The stage-specific embryonic antigens, i.e. SSEA-1, SSEA-3 and SSEA-4, are widely used for assessing stem cells' state of differentiation. Initially, the antigens were defined by the respective monoclonal antibody (37-39), but the epitopes were subsequently also structurally characterized (39-41). The expression of SSEA differs between species and was first observed in mouse

embryos and human teratocarcinoma cells (37-39). The antibody MC480 that defines SSEA-1 (37) is specific for the Le^x epitope when presented on long type 2 core chains as discussed in section 4 (40, 42). In contrast to SSEA-1, which is a lacto series glycolipid antigen (see section 1.3.2), SSEA-3 and SSEA-4 are globo series structures only found carried by lipids and not by proteins in humans (Figure 4). Hence, the SSEA-3 and SSEA-4 determinants have a globo carbohydrate core. The anti-SSEA-3 antibody MC631 (38) recognizes the internal oligosaccharide sequence (globoside), whereas SSEA-4 is defined by the antibody MC813-70 (39) that binds the terminal structure including a terminal sialic acid.

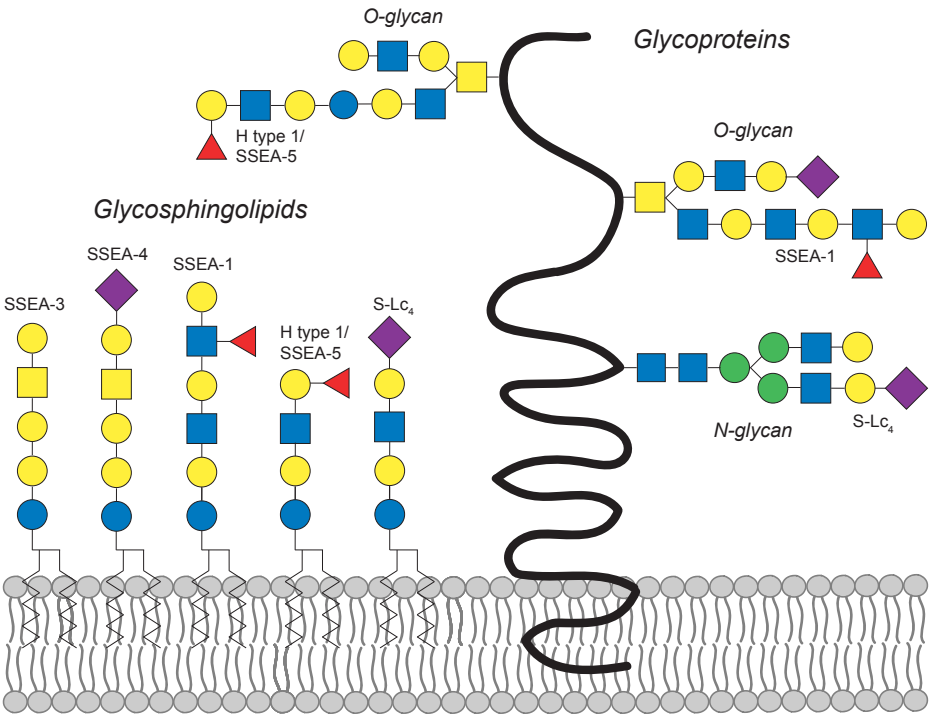

Figure 4. Cell surface carbohydrate pluripotency markers*. Simplified illustration of the antigens attached to different cell surface glycoconjungates, i.e. glycosphingolipids (GSL) and glycoproteins, integrated in the plasma membrane that mainly consists of phospholipids (grey). As shown, GSL carries only one carbohydrate chain perpendicularly oriented, compared to glycoproteins that may have several different carbohydrate chains attached. See key to glycan symbols on page vii. Abbreviations: SSEA, stage-specific embryonic antigen; S-Lc$_4$, sialyl-lactotetra.*

Expression of SSEA-1 is evident in mouse PSC (37, 40), but absent in human PSC (6, 8). Inversely, SSEA-3 and SSEA-4 are abundantly expressed in human PSC (6, 8) but not found in mouse PSC (39), consequently, establishing the typical SSEA-1$^-$/SSEA-3$^+$/SSEA-4$^+$ phenotype of hESC(36).

However, during hPSC differentiation the expression patterns of the different stage-specific antigens seemingly shift, with appearance of SSEA-1 and down-regulation of SSEA-3 and SSEA-4 (6, 36). Kannagi et al. postulated this to be a result of a possible shift in glycolipid synthesis from globo to lacto during differentiation (39).

It should be noted that hPSC lines could in theory be SSEA-3 or SSEA-4 negative due to lack of the glycosyltransferase enzyme that extends the precursor saccharides to generate globoside or terminally sialylate globopenta, e.g. in so called p individuals lacking blood group P antigen or Luke negative phenotypes lacking the Luke antigen (SSEA-4), respectively (43).

No specific developmental function has been identified for SSEA-1, SSEA-3 or SSEA-4 in humans. Although some evidence of SSEA-1/Le^x antigen involvement in the compaction process of late cleavage stage embryos in mice (44, 45), viable SSEA-1 negative mouse embryonal carcinoma stem cell lines have been derived (46, 47). Additionally, SSEA-3 negative and SSEA-4 negative individuals (i.e. p and Luke negative individuals) develop normally. Furthermore, elimination of SSEA-3 and SSEA-4 (and all glycosphingolipids) expression by culturing hESC in the presence of biosynthesis inhibitors (PDMP, ISP-1), does not effect their pluripotency characteristics (48). Consequently, it seems unlikely that these antigens have critical functions in early embryonic development or are essential for maintenance of the undifferentiated state, but may instead have roles during the differentiation process. Noteworthy, SSEA-3 and SSEA-4 are consecutively expressed in some adult tissues, e.g. erythrocytes and kidney (38, 49).

Recently, Tang et al. raised a monoclonal antibody against a surface glycan on hESC, designated SSEA-5, which binds to the blood group H type 1 epitope (Figure 4, (50)). They successfully used this antibody to remove undifferentiated cells from a heterogeneously differentiated hESC population by flow cytometry sorting and thereby reduced the frequency of teratoma formation.

The keratan sulfate antigens TRA-1-60 and TRA-1-81 are abundantly expressed on hPSC and widely used as markers for undifferentiated cells. The anti-TRA-1-60 and anti-TRA-1-81 antibodies are well established markers for pluripotency generated in mice immunized with the human embryonal carcinoma stem cell line 2102Ep (51). The exact molecular identities of the TRA-1-60 and TRA-1-81 antigens are not fully known. Presumably, anti-TRA-1-60 and anti-TRA-1-81 antibodies recognize carbohydrate epitopes carried by glycoproteins, consisting of large keratin sulphated proteoglycans (52).

Most pluripotency markers are as previously stated carbohydrate determinants. However, O´Brian et al. were recently able to generate seven new monoclonal antibodies against cell surface proteins on hPSC and identified their corresponding gene sequences (53).

1.1.1 Human embryonic stem cells

Human embryonic stem cells (hESC) are isolated from the inner cell mass of *in vitro* fertilized blastocysts approximately 5 days post fertilization as illustrated in Figure 3 (6). The blastocyst is about 0,1-0,2 mm in diameter, it contains 200-300 cells following rapid cleavage and is made up of an inner cell mass (embryoblast), which subsequently forms the embryo, and an outer layer termed the trophoblast which gives rise to the placenta. Compared to the morula stage preceding the blastocyst, hESC cells are not totipotent and consequently lack the capacity to develop into a human fetus. Human embryonic stem cells express the pluripotency genes *OCT4*, *SOX2* and *NANOG*, consistent with their origin in the inner cell mass. However, embryonic stem cells differ significantly from their *in vivo* counterparts that lack self-renewal abilities and have a hypomethylated genome (54), in contrast to the highly methylated genome of embryonic stem cells (55).

To date, more than 1000 hESC lines have been established worldwide (56). In Sweden alone more than 90 cell lines are enrolled in the Human Pluripotent Stem Cell Registry (hpscreg.eu). In the U.S., the National Institute of Health (NIH) currently has 380 accepted cell lines registered, despite prior political restrictions.

1.1.2 Human induced pluripotent stem cells

By retroviral-mediated transduction of *only* four defined transcription factors (*OCT3/4*, *SOX2*, *c-Myc* and *Klf4*) Takahashi and Yamanaka successfully managed to generate induced pluripotent stem cells (iPSC) from mouse fibroblasts in 2006 (7), and subsequently from human fibroblasts the

following year (8). Simultaneously, James A Thomson´s research group made the same accomplishment, successfully deriving hiPSC by retroviral-mediated transduction of human fibroblasts with *OCT4*, *SOX2*, *NANOG* and *LIN28* (57). Since then, a variety of different reprogramming methods have been developed and successfully used on various human cell types to alter their cellular identity (58). Besides retroviral or lentiviral gene transfer of different combinations of reprogramming genes, hiPSC can also be generated by various non-integrating adenoviral vectors, plasmids, sendai viral vectors, episomal vectors, recombinant proteins, modified mRNA, miRNA and small epigenetic modifier molecules, as well as through transposon-mediated reprogramming (58, 59).

Genome integrating methods, e.g. retroviral or lentiviral transduction, are effective, consistent and reliable reprogramming alternatives (59, 60), but are also associated with risks for compromising genome integrity and facilitating tumor formation (61) due to e.g. the introduction of the oncogene c-Myc. On the other hand, non-integrating and alternative methods have lower efficiency and are relatively resource-intensive.

Other areas of concern that may limit the therapeutic potential of hiPSC are the postulated retainment of "epigenetic memory" of their origin (62-65) and high presence of genomic anomalies such as missense mutations (66), translocations and chromosomal aneuploidy (67).

1.2 Differentiation of pluripotent stem cells

Embryogenesis is initiated by the fertilization of an oocyte, forming a single cell called a zygote. The cell divisions that follow, termed cleavage, occur without significant growth in size and result in the formation of the morula containing 16 cells (four cell divisions). These cells are totipotent and can consequently form all human cell types, as well as the extraembryonic placental cells and have the capacity to form a viable embryo. Following further cell divisions, approximately five days after fertilization, the blastocyst is formed.

Following implantation of the blastocyst, connecting the early embryo with the uterus wall, the gastrulation phase commences approximately three weeks after fertilization. During this stage of embryogenesis the embryonic germ layers, endoderm, mesoderm and ectoderm, are formed. All tissues and organs in the human body will subsequently be derived from one of these three layers.

The inner layer, the **endoderm**, forms the epithelial lining of the main parts of the gastrointestinal tract and associated glands, including the pancreas and liver, as well as the bronchial tree and alveola, urinary bladder, thyroid and parathyroid glands. The middle layer, the **mesoderm**, generates the kidneys, gonads, spleen, serous membranes, blood cells, circulatory system including the heart, lymphatic system, dermis, bone, adipose and connective tissue, cartilage, skeletal and smooth muscles. Furthermore, the formation of mesoderm leads to the development of the coelom. The outer layer, the **ectoderm**, gives rise to the central and peripheral nervous system, eyes, adrenal medulla, pituitary gland, facial cartilage, melanocytes, teeth, hair, nails, epidermis and epithelium of mouth and nose.

The differentiation potential of multipotent and adult stem cells is determined by their germ layer origin. For example, mesenchymal stem cells can mainly differentiate into osteocytes, chondrocytes, myocytes, fibroblasts and adipocytes, but preferably not into cells making up the nervous system or gastrointestinal tract. Therefore, for successful derivation it is essential to have a preconception of the differentiation state of the stem cell, i.e. totipotent, pluripotent or multipotent, as well as the putative germ layer origin to evaluate and anticipate lineage commitment.

In vitro differentiation of stem cells may occur spontaneously, especially under suboptimal culture conditions (6, 68). However, differentiation can be induced *in vitro* through modulation of the extracellular milieu, by supplementing or depleting the culturing media with different growth factors, cytokines, enzyme inhibitors and bioactive proteins such as Activin A. Presently, there are numerous different protocols for endodermal, mesodermal and ectodermal differentiation (69). Additionally, recent advances in nanotechnology have provided nanoparticles with potential to promote stem cell differentiation into various cell lineages (70).

1.3 Cell surface tissue antigens

A well-constituted and non-compromised cell surface is critical for cellular function and survival. The outer leaflet is packed with glycoconjungates facing the external milieu, i.e. the glycocalyx that covers the cell surface (Figure 5). The glycans are carried by lipids (glycolipids) or by protein (glycoproteins or proteoglycans). The plasma membrane mainly consists of phospholipids, with scattered distribution of cholesterol, sphingomyelin, glycosphingolipids and glycoproteins (Figure 4). The outer leaflet of the plasma membrane contains various tissue antigens that interact with the extracellular environment and other cells, including cells of the immune

system. A general description of the main groups of tissue antigens of relevance for this thesis is presented below.

1.3.1 Human leukocyte antigens (HLA)

The major histocompatibility complex (MHC), known as the HLA (human leukocyte antigen) system in humans, is one of the strongest immune barriers in allotransplantation. Tissue typing of the prospective donor´s and the recipient´s HLA genotype, as well as different crossmatching methods are routinely performed, since compatibility between the donor and recipient is crucial for preventing graft rejection. The HLA system consists of HLA class I (HLA-A,-B,-C) and HLA class II (HLA-DP, -DQ, -DR), among which HLA-A, -B and -DR are most important for the clinical outcome of the transplant (71).

The human *MHC* gene is located on the short arm of chromosome 6, is divided in three regions and is highly polymorphic. The class I region consists of *HLA-A*, *HLA-B* and *HLA-C* genes. The class II region consists of *HLA-DP*, *HLA-DQ* and *HLA-DR* with various possible alternative alleles in each locus and consequently a high grade of antigen polymorphism. The class III region encodes secretory proteins, such as components of the complement cascade and cytokines (e.g. TNF-α), which immunological functions are quite different from HLA class I and II. The HLA haplotype is inherited in a Mendelian fashion from each parent. Although the variations of different loci on an HLA haplotype are extensive, certain HLA haplotypes are found more frequently in different populations than expected by chance (72).

The biological role of the HLA proteins is to present processed peptide antigens for the lymphocytes of the immune system. HLA class I is expressed in variable intensity on almost all nucleated cells, but is not found on e.g. cornea, neurons of the central nervous system or adipocytes (73, 74).

In general, HLA class I presents intracellularly digested and randomly selected self-peptides for $CD8^+$ cytotoxic T cells, ultimately leading to cellular destruction trough apoptosis or lysis. HLA class II is mainly expressed on antigen presenting cells (APC) such as monocytes, macrophages and dendritic cells, although they can be up-regulated by e.g. IFN-γ on epithelial and endothelial cells. HLA class II molecules typically present phagocytized and processed peptides from proteins in the extracellular environment for $CD4^+$ T cells, which leads to activation of B lymphocytes and subsequent antibody production.

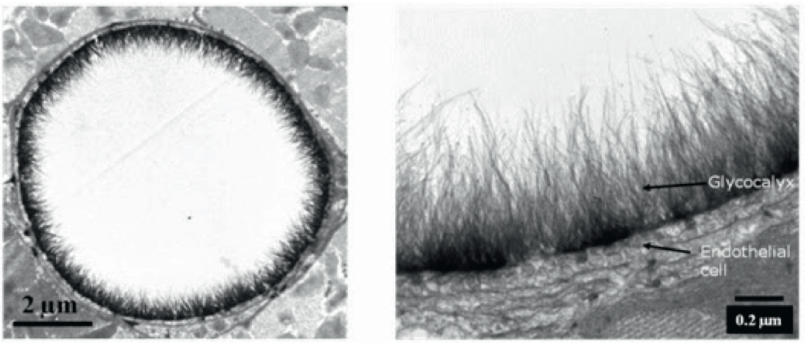

***Figure 5. Electron microscopy images of the endothelial glycocalyx in a coronary capillary** (75).*

In addition to the interaction between the peptide-HLA class II molecule and the T cell receptor (TCR), successful T cell activation also demands interaction between co-stimulatory molecules and corresponding receptors on the cell surface (Figure 10), e.g. CD80-CTLA-4, CD86-CD28 and ICAM(CD54)-LFA-1.

Consequently, HLA class I and II molecules are essential for the immunological response to non-self antigens and consequently for the adaptive immune response and T lymphocyte activation (see section 1.4).

1.3.2 Cell surface glycoconjungates

The cell surface of all human cells is covered by glycoconjungates. The meshwork, referred to as the glycocalyx, can project up to about 10 nm from the surface (Figure 5). The cell surface glycans have many known biological functions, such as physical protection (76), membrane organization, intercellular signaling, being targets for host recognition, i.e. receptors for different pathogens (77, 78), as well as self and non-self discrimination involved in autoimmunity and organ rejection. An example of a specific biologic function of a glycan is sialyl-Le^x, as the ligand of E-selectin adhesion molecule, involved in lymphocyte rolling and macrophage homing to a site of inflammation or injury (79, 80).

The biosynthesis of glycans is mainly confined within the ER and Golgi apparatus (1). Consecutive monosaccharides are added one by one in a stepwise manner through the action of different glycosyltransferases and transported to the cell surface (Figure 6). Glycoconjungates are, in contrast to proteins, secondary gene products and are not encoded directly in the genome (81), but instead a product of several different glycosyltransferases

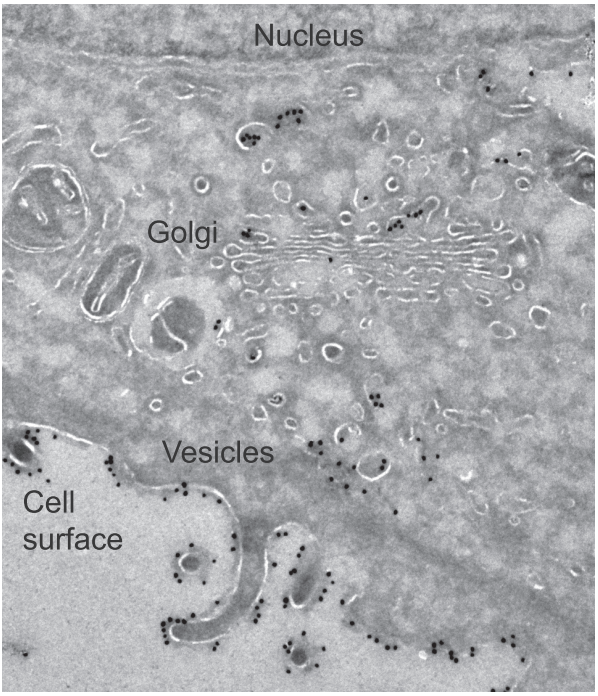

Figure 6. Electron microscopy image illustrating the main compartments for biosynthesis of cell surface glycoconjungates*. The hESC cell line SA181 stained with an antibody against the oligosaccharide determinant sialyl-lactotetra. Positive immunogold labeling (black dots) is found in the Golgi apparatus, in transport vesicles and extensively on the cell surface.*

assembling the monosaccharide moieties to elongate the glycan chain. Accordingly, a high complexity of glycans can be generated due to variations in monosaccharides, binding positions and anomeric configuration of the glycosidic linkages. Both linear and branched saccharide chains can be formed. Thus, a very high complexity of glycan structures can be produced using a limited number of monosaccharide units (1).

The membrane bound glycoconjungates are classified depending on their carriers into glycoproteins and glycolipids, respectively (Figure 4). Several carbohydrate chains can be linked to the glycoproteins while each lipid carrier only have one carbohydrate chain attached. Below are the specific properties of the membrane bound glycans described in more detail focusing on the glycosphingolipids that are the main focus of this thesis.

The nomenclature, abbreviations and symbolic presentations of glycoconjungates used in this thesis is according to "Essentials of glycobiology" 2nd edition (1).

Glycosphingolipids

Glycosphingolipids (GSL) consist of a single carbohydrate chain linked to a hydrophobic lipid part, ceramide, that is embedded in the outer part of the membrane bilayer exposing the carbohydrate chain to the outside of the cell as illustrated in Figure 4 (82-84). Glycosphingolipids are found in the plasma membrane of all human cells where they constitute from about 5% (erythrocytes) to 20% (myelin) of the membrane lipids, and up to as much as 80% of the total glycoconjungates (brain) (1). Most GSL have quite short carbohydrate chains consisting of 1-4 sugar residues, which consequently can be hidden by the relatively large glycoproteins protruding from the cell surface, prohibiting immune recognition (Figure 4). The more complex GSL with longer carbohydrate chains have a more individual specific expression exemplified by the antigen determinants of the blood group ABO, Lewis and related blood group antigen systems.

Glycosphingolipids are often assembled together with cholesterol, sphingomyelin and selected proteins into micro-domains known as lipid rafts, forming a gel phase distinct from the surrounding phospholipids, which are in a fluid state (85). The lipid rafts indirectly displace the glycoproteins and consequently expose the GSL, including the short carbohydrate determinants, and make these accessible to e.g. toxins, antibodies and microbes. The precise structure and function of lipid rafts are not fully understood (86), but several growth factor receptors, such as the insulin receptor, are localized in membrane micro-domains and their signaling is believed to be modulated by GSL (1, 87). In addition to being located in the plasma membrane, GSL can also be found in body fluids, for example short chains (1-4 sugars) in human plasma (88, 89).

Glycosphingolipids are classified as non-acid (neutral) or acid depending on the absence/presence of negatively charged saccharide moieties. This is based on the isolation procedure of the GSL components, where the non-acid and acid compounds are separated by the use of ion-exchange chromatography. Furthermore, the acidic GSL are subclassified as gangliosides containing sialic acid residues and sulphatides containing sulphate residues, respectively.

The expression of all GSL is tightly regulated during development and requires a substantial investment by the organism in gene coding of the

glycosyltransferase enzymes involved in their synthesis. Despite this, they have been shown to be dispensable in studies of GSL-depleted hESC that seemingly proliferate normally, maintain their undifferentiated phenotype and genotype, and ability to differentiate into all three germ layers (48). Furthermore, the rare p individuals (phenotype p of the P blood group system) lack blood group P (globotetra/globoside) as well as the precursor P^k (globotri) antigen (90), which constitute the vast majority of glycosphingolipids in human erythrocytes and tissues (91). Despite this, they function and develop normally except for a high incidence of spontaneous abortions noted in females with p phenotype (92).

However, the known glycosphingolipid storage diseases, e.g. Tay-Sachs, Krabbe, Gaucher and Fabry disease, are clinically significant. These rare genetic disorders lead to accumulation of GSL in lysosomes, where normally most glycans are degraded. Clinical symptoms depend on the extent of effected tissues and the range of severity. Furthermore, it has been postulated that anti-GSL antibodies are involved in certain autoimmune diseases e.g. speculated autoantibodies against gangliosides engagement in the pathogenesis of Guillian-Barré through interactions with the GSL components of peripheral nerve myelin (1). In addition, several soluble toxins (e.g. Cholera, E-coli and Shiga) and certain pathogens (bacteria and viruses) bind to specific GSL on the host cells (reviewed in (78)).

Glycoproteins

Glycoproteins have one or several carbohydrate chains attached to a protein, via a nitrogen atom to an asparagine residue (N-linked) commonly involving GlcNAc, or via an oxygen atom typically to a serine or threonine residue (O-linked) usually connecting to GalNAc (Figure 4). The biosynthesis of the carbohydrate chains is similar to the process in glycosphingolipids as described above and illustrated in Figure 6.

Glycoproteins are, unlike GSL, extensively present in the extracellular compartments as secreted proteins. However, these are not the focus of this thesis. Onwards discussion of glycoproteins will mainly refer to the cell surface integrated membrane glycoproteins.

In contrast to lipids that only carry one carbohydrate chain, glycoproteins may carry multiple and often complex carbohydrate chains that are often positioned horizontally to the plasma membrane, protruding into the extracellular milieu (Figure 4). Hence, making them more accessible antigen targets compared to GSL (1).

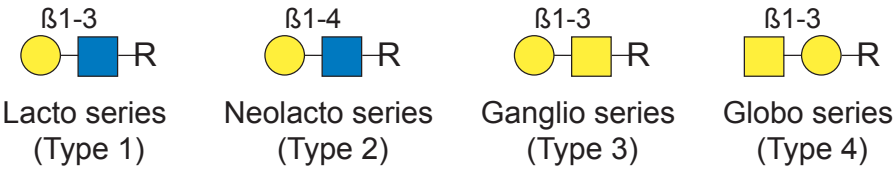

Figure 7. Classification of glycoconjungates based on their carbohydrate core structure*. Type 1-3 carbohydrate chains are found in both glycoproteins and glycosphingolipids (GSL), whereas the globo series are only found carried by GSL in human tissues. See key to glycan structures on page vii for symbolic abbreviations. R represents a protein or lipid residue.*

Glycoconjungate core saccharides

Glycoconjungates of glycolipids and glycoproteins are classified by their carbohydrate core structures (1). In GSL the major core saccharides are the lacto, neolacto, ganglio and globo series (Figure 7). The lacto series (also designated as type 1 core chain) has a β1-3 linkage between the GlcNAc and Gal residues, while the corresponding linkage in the neolacto series (type 2 core chain) is β1-4. The lacto and neolacto series compounds are present in N-glycans, O-glycans and GSL, the ganglio series are found on both glycoproteins (mainly O-linked) and GSL, while the globo series are restricted to GSL in humans.

In humans, type 1 core chains are mainly expressed in endodermally-derived tissues such as secretory organs of the gastrointestinal and reproductive tracts. Type 1 chain antigens are generally not found in ectodermal or mesodermal tissues, although there are some exceptions discussed below. On the contrary, type 2 glycans are mainly present in ectodermally- and mesodermally-derived tissues, e.g. skin, hematopoietic cells including erythrocytes and leukocytes (reviewed in (93, 94)). Both the ganglio and globo series are broadly distributed in the body, although the ganglio series are predominantly found in the brain.

The distribution of core chains is species, organ, tissue and cell dependent (87, 93), but can also differ within a tissue depending on if it is attached to a protein or lipid. For instance, in the human small intestine the blood group AB(O)H antigens bound to GSL are of type 1 core chain (95), compared to type 2 chains when carried by proteins (96).

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The peripheral structures are the main antigenic epitopes of the glycoconjugates glycan component (93). Hence, histo-blood group determinants with the same core structure essentially have the same antigenicity independently of their carrier motif.

1.3.3 Histo-blood group antigens

Blood group antigens of carbohydrate nature were initially believed to be mainly located on the cell surface of erythrocytes. However, these complex glycan antigens can be present on a variety of cells and tissues (reviewed in (97)), why the term histo-blood group is more appropriate (93) and is used throughout this thesis. Identification and characterization of the histo-blood group antigens arise from advanced analytical carbohydrate chemistry such as mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. The antigen epitope identities are based on immunostaining methods described in section 3. The AB(O)H and Lewis histo-blood group systems are the clinically most relevant antigens. Their biosynthesis pathway and structural relationship is illustrated in Figure 9 and will be discussed below.

Other relevant histo-blood group systems include the recently identified Forssman blood group system that is based on a globo core saccharide (Figure 8), hence only found in GSL in humans (98).

The histo-blood group antigens are found on the cell surface of cells, tissues and body fluids such as breast milk and saliva linked to lipids (GSL) or proteins (glycoproteins), or present as free oligosaccharides (99). They are products of the sequential action of different specific glycosyltransferases according to the same principles as biosynthesis of all glycans described above. The histological distribution of the core chain carbohydrate types are partly related to their embryonic origin, but also determined by cell type and the degree of differentiation of the cells for example within the epithelium (97).

The carbohydrate histo-blood group antigens are mainly present on epithelial cells that are in contact with the external milieu and consequently known to interact with various microorganisms. Many pathogens use cell surface carbo-

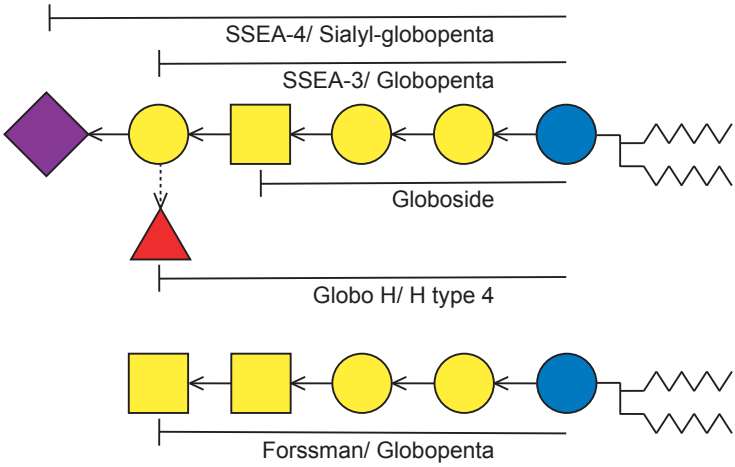

Figure 8. The biosynthesis of and structural relationship between relevant carbohydrate antigens of the globo series*. Carbohydrates of the globo series linked to a ceramide (glycosphingolipid). See key to glycan structures on page vii for symbolic abbreviations.*

hydrates as primary attachment receptors, often referred to as microbial recognition of host glycoconjungates (100). Furthermore, the antigens can be present on pathogens themselves and induce production of antibodies in the host, e.g. certain *Escherichia coli* strains express blood group B antigens (101).

AB(O)H histo-blood group system

In 1901 Karl Landsteiner demonstrated that erythrocytes express AB(O)H blood group system antigens (102). A discovery for which he was rewarded the Nobel Prize in Physiology and Medicine in 1930. During the mid 20th century, the chemical structures of the AB(O)H determinants were identified as well as various glycosyltransferases involved in their biosynthesis (93). Figure 9 illustrates the biosynthesis of the AB(O)H antigens of the lacto and neolacto series and their close structural relationship with the Lewis blood group determinants.

The AB(O)H antigens are created by stepwise addition of monosaccharaides by specific glycosyltransferases encoded by three loci, consisting of the *H*, *SE* (*Secretor*) and *ABO* genes (94). Blood group A and B antigens are elongated by different glycosyltransferases encoded in the polymorphic *ABO* locus that are inherited according to Mendel´s principles (exemplified by synthesis of blood group A type 1 and 2 in Figure 9).

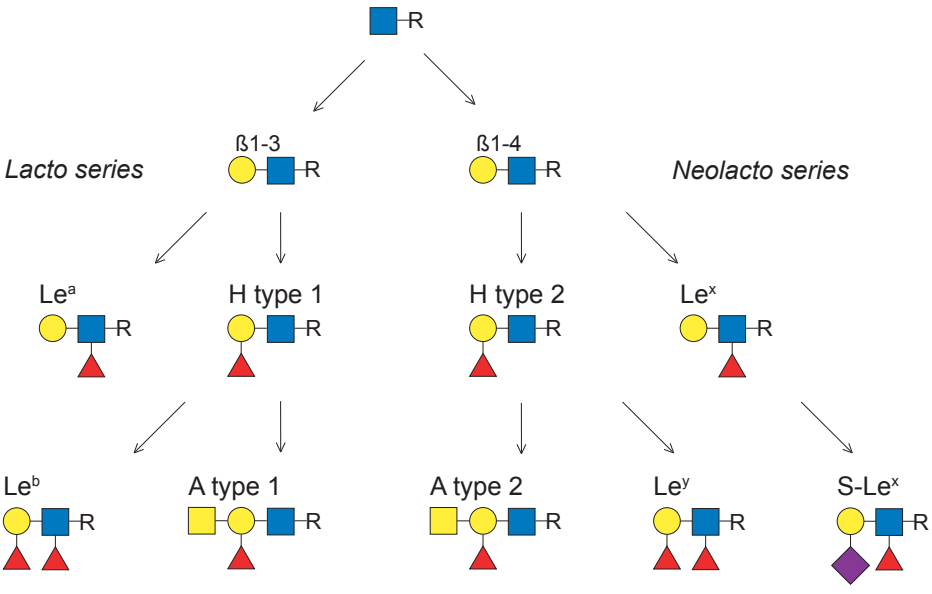

Figure 9. Biosynthesis of lacto and neolacto series AB(O)H and Lewis histo-blood group antigens*. The antigens can be carried by lipids or proteins, hence abbreviation R for residue. The arrows represent the action of each specific glycosyltransferase adding a terminal sugar to the core chains. See key to glycan structures on page vii for symbolic abbreviations.*

The blood group A and B antigens are formed by the α1-3GalNAcT and the α1-3GalT respectively, encoded by the *A* and *B* alleles of the *ABO* locus. However, the *O* allele codes for a protein that is structurally related to A and B transferases but lacks enzymatic activity (94). Consequently, O individuals only express H structures. The blood group *H* fucosyltransferase is restricted to type 2 core chains, while the *Se* gene coded fucosyltransferase can work on both type 1 and 2 based blood group core chains (1). Therefore, non-secretor individuals lack type 1 chain antigens.

Consequently, the presence or absence of particular glycosyltransferases determines an individual´s ABO blood group. Absence of certain glycosyltransferase, results in specific phenotypes such as the rare Bombay phenotype (i.e. lack AB(O)H antigens) or the more common non-secretor phenotype (i.e. absence of AB(O)H in saliva and various epithelial cells).

The distribution and frequency of blood groups varies over the world depending on ethnicity. In Sweden, about 44% of the population are blood group A individuals, 28% blood group O, 12% blood group B and 6% blood group AB (72). Furthermore, each blood group has several subgroups, of which the most common phenotypes are A1 (about 80% of all A individuals), A2 (about 19% of all A individuals), B1, O1 and O2 (72).

Type 1 chain AB(O)H antigens are besides being present on lining and glandular epithelium, also the most frequently found in excretions and body fluids. Type 2 core carbohydrate AB(O)H antigens are mainly found in skin and erythrocytes, and only weakly, if all, in endodermally-derived tissues such as the gastrointestinal tract (93). AB(O)H antigens can also be based on the globo core saccharide and consequently termed Globo H and Globo A, respectively (103).

Although the developmental or physiological function of the AB(O)H antigens remains uncertain, an individual´s ABO blood group type has important clinical implications since it is one of the strongest histocompatibility antigen barriers upon transfusion of blood products or allotransplantation of tissues and organs. The anti-A and anti-B antibodies are present in the human plasma and cause complement-dependent lysis of transfused non-compatible erythrocytes and trigger rejection of mismatched allotransplants. These preformed antibodies (mainly of IgM type) against A and B antigens, contrary to the individual phenotype, are produced early in the postnatal period and are believed to be caused by an immune response to glycoconjungates presented by microorganisms in the gastrointestinal tract with similar or identical structures as the A and B determinants (72).

Lewis histo-blood group system and related antigens

The Lewis blood group system consists of two antigens, Le^a and Le^b, based on the type 1 core chain structures. These are formed as a result of the *Se* and *Le* genes as illustrated in Figure 9 and the blood group phenotype individuals are defined as $Le(a^-b^-)$, $Le(a^+b^-)$ and $Le(a^-b^+)$. The *Le* gene codes for a fucosyltransferase adding a fucose residue in a α1-4 position to the type 1 chain GlcNAc. A corresponding fucosyltransferase adding the fucose in a α1-3 position of the type 2 core chain produces the Le^x and Le^y antigens, respectively (1). The complexity of these histo-blood group antigens is further increased by addition of sialic acid residues by the sialylated forms of Le^a and Le^x.

The Lewis blood group antigens (Le^a and Le^b) are primarily expressed on epithelial cells of tissues and organs in direct contact with the external

environment and released in soluble forms in secretions and body fluids (1). However, these antigens are also expressed on mesodermally-derived hematopoietic cells such as erythrocytes and lymphocytes, mainly through absorption of circulating GSL from plasma originating from epithelial excretion from the digestive tract (89, 94).

However, the Lewis blood group system is not as clinically relevant in transplantation and transfusion medicine as the AB(O)H blood group system due to the fact that transfused erythrocytes shed their own Lewis antigens and absorb the recipient´s. Furthermore, anti-Le antibodies are neutralized in the circulation by free Lewis antigens (72). However, analogous to other histo blood group systems Lewis antigens interact with pathogens, e.g. the attachment of *Helicobacter pylori* is facilitated by interaction with Le^b antigens (104), and in turn *Helicobacter pylori* can express Le^x and Le^y epitopes facilitating potential antibody formation (105).

1.3.4 Expression of HLA and histo-blood group antigens in hPSC

The studies characterizing the HLA expression on hPSC, are few but show consistent results. Both hESC and hiPSC seemingly have normal and variable HLA haplotypes and express HLA class I but not HLA class II antigens on their cell surface (106-109).

The studies regarding the expression of AB(O)H blood group antigens in hPSC are limited. Mölne et al. characterized the AB(O)H blood group system in nine different hESC lines, as well as hESC-derived cardiomyocyte- and hepatocyte-like cells (110). This study demonstrated expression of A and B antigens in accordance with the cell lines genotype and with different sub-cellular distributions (discussed in section 4). Differentiated cells from a blood group B hESC line, lost their expression or retained their expression of B antigen in the cardiomyocyte- and hepatocyte-like cells, respectively.

Presently, a restricted number of studies regarding the GSL compositions of hPSC have been published (111-114) and reviewed in (115). Liang et al. characterized the non-acid GSL of the globo and lacto series, as well as identified several gangliosides in hESC (111, 112). They also observed a switch of core structures of GSL during differentiation that was lineage specific, displaying a difference between ectodermal and endodermal differentiation. Additionally, Barone et al. isolated GSL from a large starting material consisting of 10^9 hESC from two different lines. This allowed characterization also of minor non-acid GSL (113), which enabled

identification of several short chain GSL, neolacto series GSL with type 2 core chains structures such as Le^x and Le^y, and in addition to previously described lacto series GSL the blood group A type 1 hexaosylceramide was found. Regarding the GSL composition in hiPSC, Ojima et al. identified a similar pattern of GSL expression as was previously found in hESC, but several GSL were not found due to low sample quantities (114). This study also verified the switch of core structure from lacto/neolacto and globo to ganglio series during ectodermal differentiation.

1.4 Immune recognition

Due to safety-related concerns with hiPSC-based therapy, mainly non-autologous hESC lines are presently used in the ongoing clinical trials. The hPSC-derived cells or tissue grafts face the risk of rejection by the recipient's immune system following the same principles as conventional organ transplants.

An active and effective immune surveillance is essential for survival in an environment surrounded by pathogens and in a body with high cell turnover, in constant change and consequent risk for mutations and tumor formation. Although being a vital protection mechanism, the immune system can become the body´s worst enemy in the setting of autoimmune disease or transplant rejection. The latter is a multifactorial process involving cellular and humoral components with innate and adaptive functions that collaborate to form an adequate immune response.

The immunological mechanisms underlying rejection of allotransplants are the innate and the adaptive immune systems, consisting of both humoral (e.g. complement factors and antibodies) and cell-mediated (i.e. leukocytes) immunity.

The **innate immune system** provides a first line of defence against pathogens and consists of everything from physical barriers (e.g. epidermis and epithelial cells) to immune cells such as neutrophilic granulocytes, mast cells, macrophages, dendritic cells and mature antigen presenting cells (APC). Besides eliminating pathogens through phagocytosis and complement lysis, the immune cells involved in innate immunity initiate and direct the adaptive immune response.

The **adaptive immune system** consists of specialized T and B lymphocytes, as well as differentiated plasma cells responsible for the humoral immunity. Hence, producing antibodies against specific antigens that mediate

complement cascade-dependent cytotoxicity. One example is non-compatible blood transfusion in which anti-A antibodies of IgM type cause complement-dependent lysis of erythrocytes expressing the corresponding blood group A antigen.

In contrary to the innate immune system that recognizes a restricted number of general microbial structures (danger signals), the adaptive immune system has a highly specific and endless microbial recognition capacity. The lymphocytes have antigen specific receptors that are central for allorecognition, provide the ability to distinguish self from non-self and subsequently generate an adequate immune response when invaded by pathogens. Briefly, the T cell receptor (TCR) recognizes a self-HLA class I or II molecule together with a presented self or foreign processed peptide (Figure 10). The latter will stimulate an immune response, through activation of cytotoxic T cells (CD 8^+) or helper T cells ($CD4^+$). In the transplantation setting the most clinically relevant alloantigens are the HLA and AB(O)H antigens.

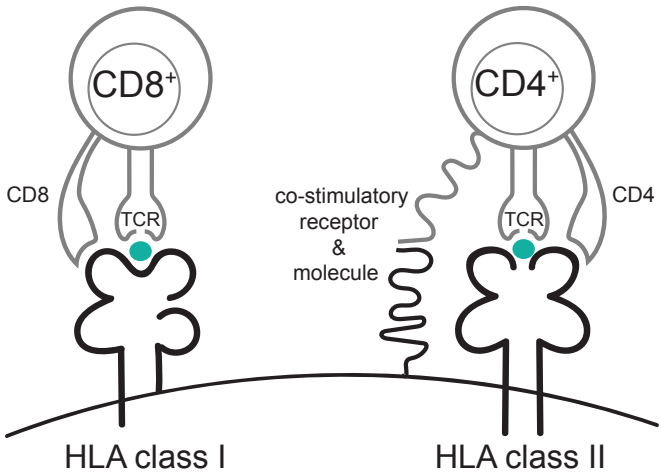

Figure 10. Processed peptides presented by HLA class I and II molecules for $CD8^+$ and $CD4^+$ T lymphocytes*. The specific T cell receptor (TCR) interacts with the peptide-HLA molecule on the cell surface. Interactions between co-stimulatory molecules and receptors are provided for successful activation of naïve $CD4^+$ T lymphocytes. CD4 and CD8 are co-receptors, which stabilize the bond between the TCR and HLA-peptide complex. The green dots represent a peptide presented on a HLA molecule.*

As previously described, the HLA surface antigens are highly polymorphic, with numerous possible antigen combinations, which complicates close compatibility between donor and recipient. HLA-matching results in less frequent and less severe rejection reactions. The AB(O)H blood group system incompatibility has less relevance on graft survival, but can trigger hyperacute rejection.

Allorecognition is initiated by $CD8^+$ and $CD4^+$ T lymphocytes that recognize either non-self HLA class I and II molecules, respectively, on donor APC (direct allorecognition) or allopeptides bound to self-HLA on the recipient's APC (indirect allorecognition) (116). The rejection process is clinically categorized in hyperacute, acute and chronic. The hyperacute occurs within minutes to hours after transplantation and is caused by preexisting antibodies in the recipient against the donor´s HLA class I or AB(O)H antigens. This type of rejection is prevented by thorough crossmatching, detecting any potential antibody reactivity against donor antigens. Acute rejection debuts within the first week(s) and is mainly a consequence of the direct T cell allorecognition pathway (71) directed by activated $CD4^+$ T cells that produce pro-inflammatory cytokines, and $CD8^+$ T cells that differentiate into cytotoxic cells mediating cell destruction. Chronic rejection occurs within months to years and is mediated mainly by indirect T cell antigen presentation that results in an alloresponse orchestrated by $CD4^+$ T cells. However, humoral mediated rejection through the formation of de-novo antibodies can occur several years after e.g. kidney transplantation.

In the case of hESC-based therapies, the main pathway for immune response is through indirect T cell allorecognition of donator-associated peptides, e.g. processed donor HLA molecules presented as alloantigens by the HLA class II on the recipient´s APC, which subsequently activate an alloresponse involving $CD4^+$ T cells. Successful activation of naïve T cells requires a sequential signaling process consisting of TCR recognition, co-stimulatory molecule signaling and cytokine production (71). In this context, the most important co-stimulatory molecules are CD80 (B7-1) and CD86 (B7-2) on APC, which are recognized by CD28 on the T cell surface (Figure 10). However, if the hPSC or their derivatives express HLA class II they would potentially elicit a direct T cell allorecognition pathway, i.e. the TCR recognizes the peptide-allogeneic HLA (I or II) complex and mistakes it for a self-MHC with foreign peptide. The latter would require the expression of HLA class II as well as co-stimulatory molecules on the hPSC-derived cells (71).

2 AIMS

This thesis is based on explorative studies with the general aim to expand the present knowledge of cell surface tissue antigens expression in human pluripotent stem cells (hPSC) and their derivatives, with the potential use in both basic science as well as in future clinical application.

The specific aims were to:

- Structurally characterize the glycosphingolipid constitution of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) to identify possible hPSC-specific structures that can be used as selection tools as well as markers of pluripotency.

- Characterize the potential immunogenicity of hPSC induced by their expression of various cell surface tissue antigens, e.g. HLA and AB(O)H histo blood group antigens, which can trigger an adverse immune response in the recipient.

- Study the alterations in cell surface antigen expression during endodermal, mesodermal and ectodermal differentiation of hPSC into hepatocyte-like cells, cardiomyocyte-like cells and neural stem cells, respectively.

3 MATERIALS AND METHODOLOGICAL CONSIDERATIONS

The studies included in this thesis investigate the cell surface antigen phenotype of several human pluripotent stem cell lines and differentiated cells derived from these cells. The analytical methods used include various immunostaining and biochemical analytical techniques as well as genotyping (Figure 11). The relevant cell lines, culturing conditions and analytical techniques are described below. Details regarding the protocols are found in Paper I-III.

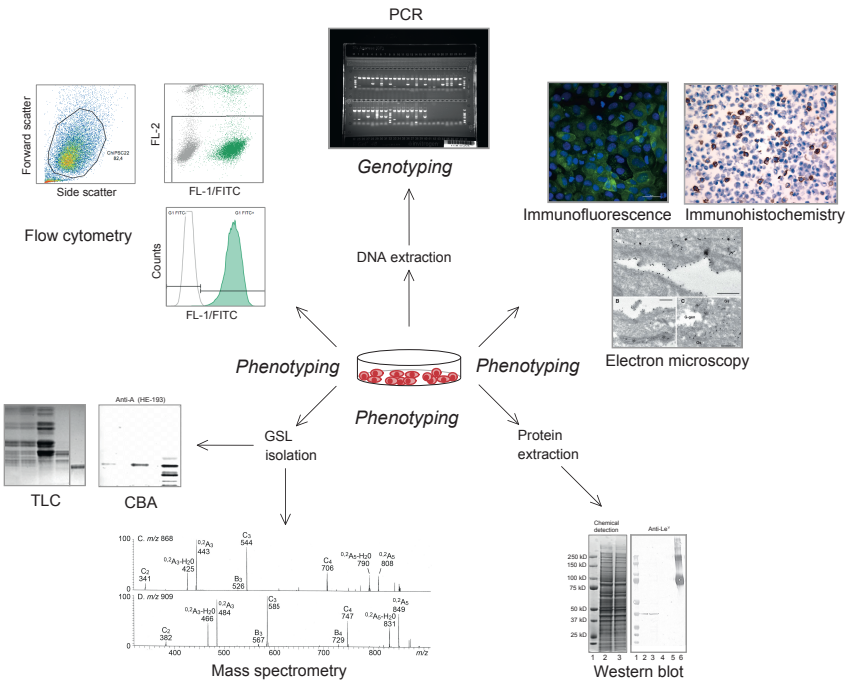

Figure 11. Summary of the analytical techniques used to characterize hPSC and their derivatives*. Genotyping was performed by polymerase chain reaction (PCR) methods. Phenotyping was performed with different immunostaining methods; flow cytometry, immunochemistry techniques with various detecting methods. Glycoproteins were analyzed by Western blot. Glycosphingolipid (GSL) were isolated and purified, separated by thin-layer chromatography (TLC), followed by immunostaining with chromatogram binding assay (CBA) and structural analysis with mass spectrometry.*

3.1 Human pluripotent stem cell lines

The human pluripotent stem cell lines used in this thesis are listed in Table 1 and were provided by Takara Bio Europe AB (former Cellartis AB and Cellectis Inc.) after in-house quality control and verification processes described below.

Cell line	Cell type	Culturing conditions	Derivatives	Ref	Paper
Val9	hESC	Xeno- and feeder-free	Hepatocyte-like	(117-119)	III
SA121	hESC	Feeder-free	Hepatocyte-like	(118, 120-125)	I, II, III
SA181	hESC	Feeder-free	Hepatocyte-like	(118, 120, 124, 126)	I, II, III
SA001	hESC	Mouse fibroblast feeders		(127, 128)	I
SA002	hESC	Feeder-free	Cardiomyocyte-like	(120-122, 124, 125, 128)	I
AS038	hESC	Feeder-free		(120, 122, 124)	I
SA348	hESC	Mouse fibroblast feeders		(127)	I
SA461	hESC	Mouse fibroblast feeders		(118, 121, 127)	I
ChiPSC4	hiPSC	Feeder-free	Hepatocyte-like	(118, 126)	I, II, III
ChiPSC7	hiPSC	Feeder-free		(118)	I
ChiPSC9	hiPSC	Feeder-free		(118)	I
ChiPSC15	hiPSC	Feeder-free		(118)	III
ChiPSC22	hiPSC	Feeder-free	Cardiomyocyte-like	(118)	III
CI2-iPSC	hiPSC	Feeder-free	Neural stem cells	(129, 130)	II
CI3-iPSC	hiPSC	Feeder-free		(129, 130)	II
P11012	hiPSC	Feeder-free		(118)	I

Table 1. Presentation of all the human pluripotent stem cell lines and their derivatives characterized in the studies*. Abbreviations: hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; Ref, references of derivation and characterization of the hPSC lines.*

The hPSC lines and their culturing conditions were controlled after derivation, propagation, as well as after thawing and passaging procedures (127). Briefly, the quality control program used involves safety screening for infectious pathogens (e.g. mycoplasma, bacteria and human viruses), evaluation of chromosomal status (i.e. FISH or karyotype analysis), morphology, telomerase activity (quantitative real-time PCR of *hTERT* and *OCT-4*) and pluripotency assessment. The pluripotency was evaluated by phenotypical characterization (i.e. immunohistochemistry and flow cytometry) of various pluripotency markers, such as OCT4, TRA-1-60, TRA1-81, SSEA-4 and SSEA-1, as well as establishing *in vivo* (immune-deficient mice) and *in vitro* differentiation of hPSC into all three embryonic germ layers (120, 122, 124, 127). Seemingly, the genomic integrity stays intact after repeated freeze-thaw procedures and prolonged continuous *in vitro* culturing (121), as well as under large-scale propagation in feeder-free culturing systems (122).

In general, the hPSC cell populations were homogenous in their morphology and phenotype upon repetitions and between passages, with some exceptions discussed below.

Human embryonic stem cell lines

All hESC lines used in our studies (except for Val9) were derived from surplus human embryos from clinical *in vitro* fertilization (IVF) treatment at Sahlgrenska University Hospital (prefix SA-, Gothenburg, Sweden) and Uppsala University Hospital (prefix AS-, Uppsala, Sweden) (120). Briefly, the donated embryos were cultured into blastocyst stage and extracted by either pronase treatment or consecutive immunosurgery. All hESC lines were cultured on feeder layers of mitomycin-C inactivated mouse embryonic fibroblasts upon establishment. However, following recent years technical advancements, all hPSC lines are presently cultured in feeder-free systems (Cellartis® DEF-CS™ 500 Culture System, Takara Clontech, Y30010).

The GMP-grade hESC line Val9 was obtained from the National Stem Cell Bank of Spain and used in the European Commission project “InnovaLiv” (131). The cell line was derived as previously described (10, 117, 118, 120), established and maintained under completely xeno-free conditions (117).

Human induced pluripotent stem cell lines

The hiPSC lines ChiPSC7, ChiPSC9, ChiPSC15 and ChiPSC22 were derived from human dermal fibroblasts using retroviral programming (118, 126). Briefly, retroviral transduction with *OCT4*, *SOX2*, *KLF4*, *LIN28* and *L-MYC* was performed in feeder-free culture systems (Cellartis® DEF-CS™) (118).

Cell line ChiPSC4 was established by transduction of human fibroblasts with a recombinant lentiviral vector encoding mouse *OCT4*, *SOX2*, *KLF4* and *L-MYC* and cultured on feeder layers of mitomycin-C inactivated mouse embryonic fibroblasts (126). Following initial derivation, the ChiPSC4 cells used in our studies have been cultured in feeder-free culturing systems.

Cell line P11012 was derived from adult dermal fibroblasts by episomal reprogramming in a feeder-free culture system (Cellartis® DEF-CS™) (118). Likewise, the hiPSC lines CI2 and CI3 were generated using non-integral episomal reprogramming technique (129) on adult human adipocytes isolated by liposuction from abdominal adipose tissue (130).

3.2 *In vitro* differentiation of hPSC

Differentiation of hPSC is accomplished by manipulation of the extracellular milieu, by using customized culturing medium compositions, supplemented or depleted with various growth factors, cytokines, transcription factor inhibitors/stimulators, enzyme inhibitors, as well as through alterations of culture plate coatings. The protocols used in our studies are schematically illustrated in Figure 11, described in Paper I-III and in the text below.

Endodermal differentiation

Endodermal differentiation into hepatocyte-like cells was conducted by Takara Bio Europe AB by applying the Cellartis® Definitive Endoderm differentiation kit (Takara Clontech, Y30035), followed by Cellartis® Hepatocyte differentiation kit (Takara Clontech, Y30050, (128)). In short, the hPSC culture is exposed to glycogen synthase kinase 3 (GSK3) inhibitors and Activin A for two days, followed by frequent changes of media supplemented by Activin A. Definitive endoderm (DE) is obtained at day 8 of differentiation. Subsequently, the DE cells are reseeded at 100 000-150 000/cm^2 to facilitate propagation of pure DE populations. Further differentiation is promoted by the specialized media composition that includes hepatocyte growth factor (HGF), epidermal growth factor (EGF) and Oncostatin M, as well as a number of non-disclosed factors included in the Cellartis® Hepatocyte differentiation kit.

The validation and assessment of the end-product is accomplished through evaluation of the cytochrome P450 (CYP450) activity and characteristic morphology, as well as expression of hepatocyte markers such as alpha-1-antitrypsin, albumin, cytokeratin 18 and glutathione S-transferase A1 (118, 126, 128, 132).

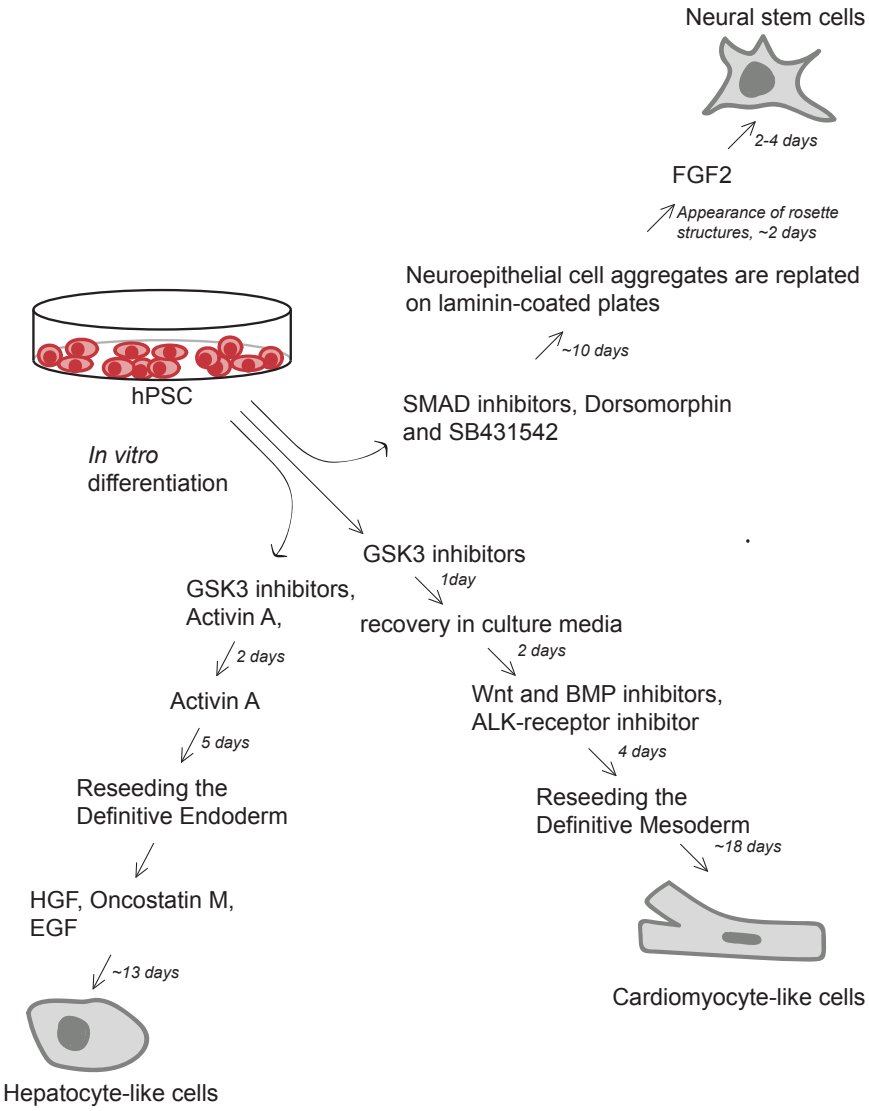

Figure 12. Schematic illustration of the key elements and steps of in vitro differentiation of hPSC used in the studies. *Illustrating the endodermal, mesodermal and ectodermal differentiation of hPSC into hepatocyte-like cells, cardiomyocyte-like cells and neural stem cells, respectively. In addition to specific supplements inducing differentiation, the corresponding culture medium used is optimized for each differentiation step and cell type. Abbreviations: hPSC, human pluripotent stem cells; FGF2, fibroblast growth factor-2: GSK3, glycogen synthase kinase 3; HGF, hepatocyte growth factor; EGF, epidermal growth factor; BMP, bone morphogenetic proteins; ALK, anaplastic lymphoma kinase.*

Mesodermal differentiation

Mesodermal differentiation from hPSC into cardiomyocyte-like cells (Cellartis® Cardiomyocytes, Y10075) was performed by Takara Bio Europe AB (Gothenburg, Sweden). Briefly, induction is initiated by exposing the hPSC culture for GSK3 inhibitors that stimulate Brachury expression (similarly to endodermal differentiation), followed by a recovery period in enriched culture media. The cell population is then cultured in the presence of Wnt and BMP (bone morphogenetic proteins) inhibitors and ALK (anaplastic lymphoma kinase)-receptor inhibitor for 4 days. The subsequent definitive mesoderm is reseeded to facilitate the propagation of pure cultures. Following culturing in appropriate cardiomyocyte maintenance media the cardiomyocyte-like cells were obtained on day 26 of differentiation.

The differentiated cells spontaneously start beating around day 11-13 of differentiation, confirming that cardiomyocyte-like cells are obtained (125). Further cell characterization includes flow cytometry analysis of cTroponin expression, as well as evaluation of the presence of F-actin by immunofluorescence. According to the manufacturer the cardiomyocyte-like cells derived through this protocol show an adequate and anticipated dose-dependent response to potassium and calcium channel blockers, e.g. E4031 and Verapamil.

Ectodermal differentiation

The ectodermal differentiation of hiPSC lines CI2 and CI3 into neural stem cells (NSC) was performed according to a protocol by Shi et al. (133) as described in Paper II. Briefly, confluent hiPSC cultures were obtained in a feeder-free Cellartis® DEF-CS™ culture system. Neural differentiation was initiated by changing the culturing media and adding two SMAD inhibitors (Dorsomorphin and SEB431542). Following neuro-epithelial sheet formation the cells were enzymatically dissociated and aggregates were reseeded on laminin-coated plates (Figure 12). Subsequently, rosette formations of NSC appear, whereupon fibroblast growth factor 2 (FGF-2) was supplemented to the media to facilitate propagation of the NSC population.

3.3 HLA and ABO Genotyping of hPSC

Genotyping of hPSC lines was performed by polymerase chain reaction (PCR) methods.

HLA typing

HLA-typing was performed with reverse-Sequence-Specific Oligonucleotide (SSO) technique using a bead-based method (Luminex®), i.e. LABType®SSO (One Lambda Inc.) (134). Briefly, the target DNA sequence is amplified by PCR using a group-specific primer for the HLA locus of interest. The PCR product is biotinylated and consequently detectable using R-Phycoerythrin-conjugated Streptavidin (SAPE). Following denaturation, the PCR product is hybridized to complementary SSO probes bound to fluorescently coded microspheres. Hence, the amplified DNA bound to SSO probes on the distinguishable microspheres is tagged with SAPE. Subsequent flow cytometry analyses provide quantitative results of the amplification as well as HLA assignment, i.e. identifying the individuals HLA class I and II alleles.

ABO typing

ABO-typing was performed by a PCR-Sequence-Specific Primer (SSP) method according to a protocol developed by Drowning and Darke (135). Extracted DNA was exposed to a mixture of nucleotides (dNTPs) and DNA polymerase, aliquotted into microtitre trays containing a specific primer set, followed by consecutive PCR amplification. The PCR products were thereafter electrophoresed through an agarose gel containing the DNA-binding dye ethidium bromide that visualizes the amplified ABO alleles when exposed to ultra violet light.

3.4 Characterization of cell surface antigen expression in hPCS and their derivatives

The hPSC lines antigen expression and cellular distribution was studied by various immunostaining and analytical biochemical techniques (outlined in Figure 11). Detection principles used in our studies include fluorescence methods consisting of flow cytometry and immunofluorescence, whereas immunohistochemistry and western blot were analyzed with colorimetric detection. Furthermore, immunogold procedures were used to demonstrate primary antibody binding when analyzed by electron microscopy. Analysis of the glycosphingolipid composition with chromatogram binding assay

(CBA) methods was detected by colometric or autoradiographic methods (^{125}I-labeled secondary antibody).

As a general remark, all *in vitro* analytical techniques have the disadvantage that the cells are not studied under normal physiological conditions. Factors such as temperature, the artificial extracellular milieu and mechanical stress during washing and staining procedures have to be optimized and considered when analyzing the results.

The following descriptions of the methods in the sections below are summarily and conceptually formulated. See Papers I-III for detailed experimental protocols.

3.4.1 Antibody-related considerations

All immunostaining techniques are dependent on the biological reagents used. Therefore, detailed knowledge of the antibodies immunoglobulin class and subclass, intended or validated epitope specificity and cross-reactivity is essential. In Papers I-III we mainly used commercial monoclonal IgG or IgM antibodies, although single polyclonal antibodies as well as in-house produced antibodies were occasionally used. See Supplemental Tables in Papers I-III for detailed antibody descriptions.

Prior to the introduction of monoclonal antibodies in the mid-1970s (136), polyclonal sera/antibodies and lectins were the biological reagents used for identification of different antigens. However, with the entry of monoclonal antibodies it became possible to establish hybridoma antibodies that distinguish variants of e.g. AB(O)H antigens with different core structures.

Antibodies do not distinguish between protein- and lipid-linked carbohydrate antigens. Most carbohydrate-reactive antibodies are directed against the terminal carbohydrate structures. However, this is not the case for example for the anti-SSEA-3 antibody (MC631) that recognizes the internal structure (GalNAcβ3Galα4Galβ4) of the SSEA-3 antigen whether or not it is sialylated (39). Thus, this antibody does not discriminate between SSEA-3 and SSEA-4 antigens (Figure 8). Furthermore, anti-blood group A antibodies generally recognize the terminal trisaccharide determinant of the A antigen (Figure 9). However, immunoglobulins and antibodies that require a larger part of the antigen including the core chain saccharides exist in humans (137, 138) and can be obtained by hybridoma techniques (93, 139), respectively.

Although monoclonal antibodies have a higher specificity than other biological reagents, they are surrounded by a number of concerns that need to

be addressed and considered. Structural characterization of the target antigen binding epitope and assessment of the antibody´s affinity to the designated epitope are of importance. Other areas of interest are the analytical conditions such as temperature, type of buffer used and cell/tissue treatment. Furthermore, the primary and secondary antibody must be matched correctly with regards to analyzing technique, type of cell/tissue analyzed and in which animal the primary antibody is raised as well as its animal reactivity.

Commercial monoclonal antibodies are not always as well characterized and validated as for example the anti-SSEA antibodies. The exact structure of the epitope to which the antibody reacts is often unknown. Furthermore, the commonly occurring phenomenon of cross reactivity between structurally related antigens is another area of concern. The specificity of an antibody relies on the uniqueness of the epitope it binds to. Antibodies against carbohydrate structures show a high frequency of cross-reactivity (140), why it is desirable to use a panel of well-characterized antibodies when analyzing the expression of glycans. Non-specific background is due to unintended binding to unrelated but similar epitopes. However, here the antibody´s affinity will be lower than for the specific target epitope and can consequently be reduced by further dilution of the antibody. Blocking agents (e.g. BSA) an also contribute to the prevention of these low affinity interactions. Background and unspecific noise caused by the secondary antibody can be assessed by preparing a negative control consisting of cells stained exclusively with the secondary antibody. Furthermore, in flow cytometry analysis isotype controls can be used to evaluate the non-specific antibody staining to cellular components of a particular isotype conjugate and specific fluorochrome (141).

3.4.2 Flow cytometry

Flow cytometry (FC) is a tool for rapidly identifying subsets of cells in a heterogeneous cell population, as well as for characterization of cell surface antigen expression. Presently, FC instruments have the ability to simultaneously analyze fluorescence signals from more than 10 different fluorochromes (142), in addition to measuring light scatter estimating cell size and granularity. However, recent technology advancements entail new complexity when analyzing and interpreting FC data, including considerations of spectral overlaps demanding fluorescence compensation, advanced set-ups and complex sorting and gating procedures. The FC used in our studies consists of single-colored analysis of relatively homogenous populations viable, non-permeabilized and mainly unfixed cells. This

experimental design minimizes the complexity and consequent difficulties with data interpretation as well as potentially succeeding errors.

The FC experiments were in general repeated three times and the results were highly reproducible within, and to a large extent also between, the investigated cell lines of the same differentiation stage. Typically, duplicate samples were prepared and all cell lines and consecutive differentiation days were examined on three separate occasions with weeks to months between experiments, verifying the results generalizability for the examined cell line.

The principle of flow cytometry analysis outlined in Figure 13 includes light scattering, light excitation and emission of fluorochrome molecules, generating specific multi-parameter data.

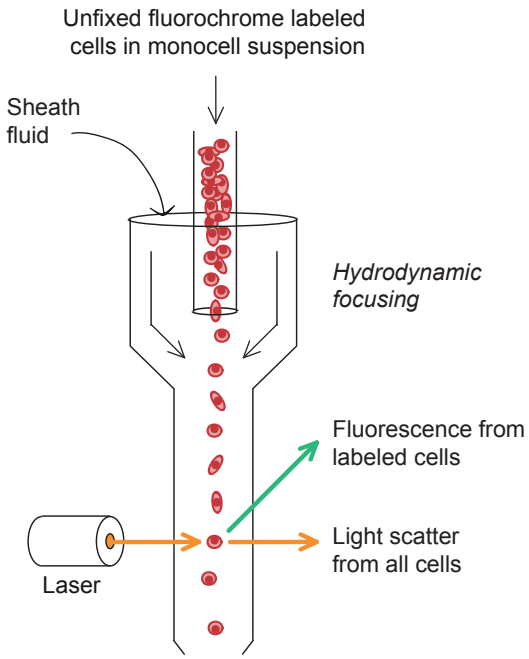

Figure 13. Schematic illustration of flow cytometry*. A monocell suspension of unfixed cells is "forced" separately into a laser beam. Consequently, fluorochromes of the positively stained cells are excited and the fluorescence is emitted at a specific detectable wavelength. Furthermore, scattered light is detected from all cells and assess the relative size (Forward Scatter) and granularity (Side Scatter) of the cells.*

Briefly, flow cytometry uses hydrodynamic focusing of monocell suspensions stained with fluorochrome-conjungated antibodies, forcing the cells to pass separately through a laser beam at high speed, enabling rapid assessment of millions of cells consecutively. Laser excitation of the fluorochromes emits fluorescence at a specific wavelength, which is identified and distinguished by the FC detectors. Furthermore, all cells exposed to the laser beam emissions light scatter, regardless of antigen expression and consequent antibody reactivity. Light scatter in the forward direction is collected by the Forward Scatter channel (FSC) and is correlated to the relative size of the cell (x-axis in Figure 14A). Light measured at a right angle to the laser beam is collected by the Side Scatter channel (SSC), providing information of the relative granularity of the cell (y-axis in Figure 14A).

In general, we did not experience any apparent difficulties with high non-specific binding of antibodies of IgM type compared to IgG subclass, as reported by others (143). The main fluorochrome used was fluorescein isothiocyanate (FITC), which has an excitation and emission spectrum maximum wavelength around 488-520 nm (144), generating a green color detectable in the FL-1 channel.

To enable optimal possibilities to compare results between the various techniques used, we mainly used non-conjugated primary antibodies. The secondary antibodies of relevant immunoglobulin class and animal specificity were labeled with FITC. The cytometer was calibrated daily using BD CaliBRITE™beads to set fluorescence compensation, adjust instrument settings, and check instrument sensitivity. As negative controls we used unstained cells, cells incubated only with the relevant secondary antibody and adequate isotype controls, to evaluate autofluorescence, background staining and account for day-to-day variations. Positive controls were established cell surface pluripotency markers. The staining, incubation and short-term storage of the cells were performed at 4°C. Analysis was conducted with a FACSCalibur^{TM} flow cytometer, un-gated acquisition was made by the CellQuest^{TM} software on a logarithmic scale and analysis and gating procedures were executed by the FlowJo (v10.1.r5) software (Figure 14A-C). The forward (FSC) and side light (SSC) scatters were adjusted to exclude duplicates, debris and dead cells (Figure 14A). The latter was further enhanced by using a propidium iodide (PI) exclusion method, i.e. staining the cells with PI and consequently identifying and excluding the positive cell population detected in the FL-2 or FL-3 channels (Figure 14B).

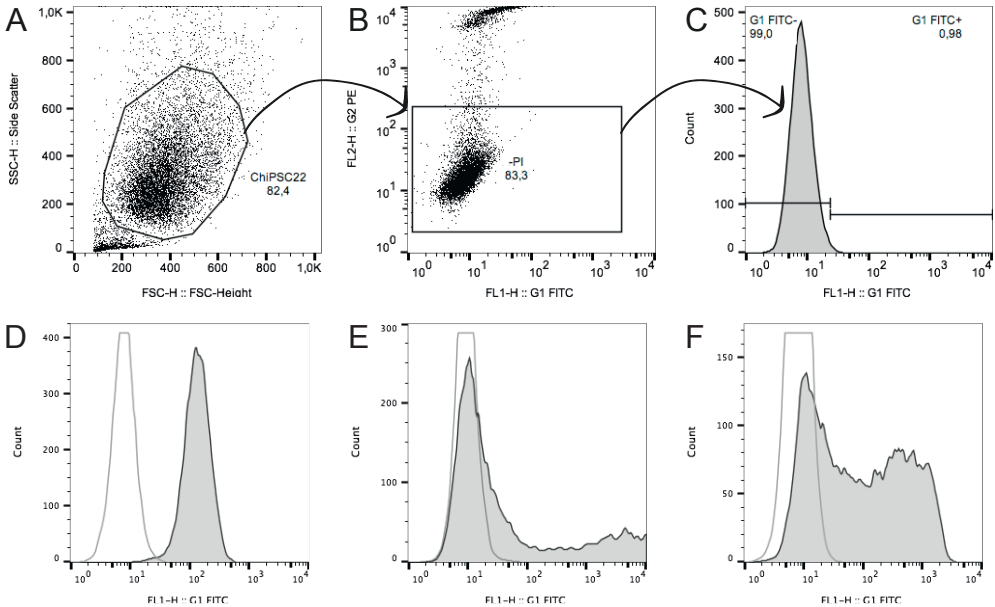

***Figure 14. Flow cytometry analysis and gating procedures**. (**A**) A generous gate is set around the cell population in the forward scatter (FSC) vs. side scatter (SSC) dot plot, excluding debris, dead cells and doubles. (**B**) Subsequently, the propidium iodide (PI) stained cells are detected in the FL-2 channel and excluded by setting a gate around the negative population. (**C**) Consistently, the cut-off value for separating negative from positive cells was set when the gate include 99% of the negative control sample (grey histogram), represented by the horizontal bar. (**D**) The filled grey histogram represents cells stained with a specific FITC-conjugated antibody. The transparent grey curve consists of a negative control. Hence, all cells express the investigated antigen. (**E**) The filled grey curve represents cells stained with a specific FITC-conjugated antibody where only a subpopulation of cells are positively stained at high intensity, whereas the majority of the cells are negative. (**F**) Illustrating one of the difficulties with interpretation of FC data. Does this histogram reflect a subpopulation of positive cells or do in fact all cells express the antigen in question although with low density?*

Since PI is a polar membrane impermeable dye that binds double stranded DNA, it only stains damaged, non-intact and non-viable cells with compromised membrane integrity, whereas it generally is excluded from viable and intact cells. Therefore, excluding the PI positive population limits the influence potential non-specific antibody binding to dead cells and exposed intracellular components could have on the acquired data, as well as providing a viability test for the cell population in general.

When analyzing and presenting FC data, histograms and dot plots are often used (Figures 14). Histograms display a single parameter, e.g. fluorescence, with the density of the signal recorded on the x-axis and the number of cells with corresponding intensity on the y-axis (Figure 14C-F). Dot plots (Figure 14A-B) on the other hand show distribution of cells in terms of two parameters, e.g. FSC vs. SSC or FITC vs. PE (phycoerythrin, emits red color upon excitation at 488nm). Determination of positive vs. negative cut-off points were set to include 99% of the negative control cells (Figure 14C) and was evaluated for each immunoglobulin class and animal specificity separately. In general, the isotype controls and cells stained with only secondary antibody emitted negligible and comparable fluorescence, indicating modest non-specific binding and autofluorescence.

When analyzing, interpreting and presenting our data we generally used frequency of positive cells measured in percentage, rather than mean/median fluorescence intensity (MFI). This strategy has been postulated to be more relevant for assessing pluripotency (145). Consequently, the cell surface antigen expression characterized in Paper I-III is defined in bivariate terms of positive or negative as a semiquantitative measure. This approach was suitable for our objectives of qualitative evaluation of various cell line types and identification of possible subpopulations. Noteworthy, the intensity of fluorescent signal is proportional to the amount of antibody bound per cell, and hence, related to the number of epitopes on the cell surface.

One challenge we encountered when interpreting the data was to distinguish a positively stained subpopulation of cells (Figure 14E) from a low intensity staining of all cells as illustrated in Figure 14F. We addressed this issue by performing complementary immunohistochemistry to further characterize the subcellular distribution of the antigen.

As a general remark, FC is a sensitive technique that detects antigens on the cell surface of non-fixed cells. However, the analysis is performed at +4°C, which may result in altered accessibility of the antigens due to a more rigid topography of the cell membrane at this non-physiological temperature preventing, for example, formation of lipid rafts (86). Consequently, certain antigens may be non-detectable, which can cause false negative results.

3.4.3 Immunochemistry

Immunochemistry includes antibody-dependent techniques that label the epitopes of interest in cells, i.e. immunocytochemistry (IC), and tissues, i.e. immunohistochemistry (IH). We used immunochemistry techniques as complementary analysis methods to verify flow cytometry results and determine subcellular distribution of antigens, as well as to demonstrate intracellular antigen expression.

Although we studied cultured cells, the preparation method used in Papers I and III included embedding of mechanically removed intact monolayers of cells or cell pellets originating from monosuspension of cells in paraffin according to protocols for tissue samples, why we have consistently referred to the method as **immunohistochemistry**. The preparation and staining procedures used took place at room temperature and have previously been described in detail (110), as well as in Papers I and III. In general, live cells were harvested enzymatically by trypsin or mechanically scraped from the culture flask as monolayers. Regardless of releasing technique used, the cells were fixed in buffered paraformaldehyde, followed by dehydration, paraffin embedding and consecutive sectioning into 4-µm slides. Antigen retrieval was performed by microwave treatment and endogenous peroxidase activity was blocked by immersion in peroxidase-blocking solution. Consecutive immunostaining was executed in a computer-assisted Autostainer Plus processor (Dako) or manually using a Vectastain Elite ABC kit (Vector Laboratories, BioNordika). Finally, slides were stained with haematoxylin and permanently mounted under cover slips and analyzed by light microscopy.

In Paper II we used the term **immunofluorescence**, referring to immunocytochemistry methods detected by immunofluorescence, i.e. by emission of light from an excited fluorochrome. This method is further referred to as IF and described in detail in Paper III. Briefly, the cells were enzymatically dissociated, replated on laminin coated chamber slides and cultured overnight. Subsequently, the cells were fixed by paraformaldehyde, permeabilized and blocked. Following immunostaining and counterstaining of the nuclei with Hoechst 33258, the slides were mounted and analyzed by fluorescence microscopy.

Transmission **electron microscopy** (TEM) following immunocytochemistry preparations, i.e. immunogoldstaining of cryosections, is a method for high-resolution imaging of the localization and subcellular distribution of a specific antigen and was used in Paper I (Figure 6). Electron microscopes

(EM) have electromagnetic lenses, compared to optical lenses found in a conventional light microscope that focuses a high velocity electron beam instead of visible light. Since electrons are absorbed by atoms in the air, electron microscopy demands ultrahigh vacuum conditions. Hence, the preparation process is demanding, requires high level of expertise and is described in detail in Paper I. Generally, cells do not tolerate these preparatory techniques, especially the absence of water that can cause destruction of macromolecules. Therefore, cryosections that allow examination of hydrated cells, are prepared. The freezing procedure is done rapidly to prevent the water from crystallizing and to preserve a vitreous form that does not expand when solidified. In scanning electron microscopy (SEM), an intense and sharply focused electron beam scans rapidly over a sample that is coated with a heavy metal. The technique allows a seemingly three-dimensional view of the cell surface of unsectioned cells (Figure 2).

In general, immunochemistry analysis provides invaluable information regarding the subcellular localization of expressed antigens. However, the chemical solutions used in the fixation and deparaffinization processes can cause alterations in epitope-structures of both carbohydrates and proteins, resulting in weak staining and false negative results (146). In general, we found weaker staining in IH compared to FC analysis although the outcome of the two antibody-dependent methods showed a high level of consistency.

3.4.4 Immunostaining of proteins and glycosphingolipids

Western blot (WB) is a method to detect a particular protein in a complex mixture, by combining the resolving power of gel electrophoresis, the specificity of antibodies with the sensitivity of enzyme assays. Briefly, a crude protein extract was enzymatically prepared, separated on a Bis-Tris gel (NuPAGE) by electrophoresis, transferred onto a nitrocellulose membrane and subsequently incubated with the primary antibody with affinity for the antigen of interest. This was followed by staining with corresponding secondary antibody covalently linked to alkaline phosphatase (AP), which catalyzes a chromogenic reaction. After adding substrate the subsequent precipitate was detectable and the antigen containing protein band was visualized.

Thin-layer chromatography (TLC) separates GSL according to their size, identifies compounds in a mixture and determines the purity of a substance. The technique is performed on an aluminum- or glass-backed silica gel (stationary phase) with a solvent system consisting of

chloroform/methanol/water (mobile phase). Competition between the stationary and mobile phase results in separation of individual compounds depending on their polarity, i.e. number of monosaccharides and degree of hydroxylation of the ceramide. Detection of the GSL are made by anisaldehyde that colors all GSL green (147) or resorcinol reagent that only stains sialic-acid containing GSL (gangliosides) purple, distinguishing them from sulphatides in the acid GSL fractions (148). As a complement, the use of borate-impregnated plates enables discrimination between glucose and galactose containing monohexosylceramides (149).

The TLC plates are useful for monitoring the GSL purification process (see below), provide qualitative and quantitative information of the GSL composition and partial structural data by comparing them to structurally well-characterized reference GSL (150). Furthermore, TLC plates can be analyzed by an immunostaining modality, i.e. **chromatogram binding assay** (CBA), characterizing the carbohydrate chain length and epitope structure binding the monoclonal antibodies used (151). The CBA method used in our studies is described in Paper I-III and the reactions were detected by colometric (alkaline phosphate-conjugated secondary antibody and nitro blue tetrazolium chromogenic substrate) or autoradiographic methods (^{125}I-labeled secondary antibody).

3.4.5 Glycosphingolipid isolation and structural characterization

Isolation of glycosphingolipids

Isolation of a pure glycosphingolipid (GSL) fraction free from contaminating lipids involves a large number of sequential steps. The procedure is outlined in Figure 15 and based on a protocol developed by Karlsson (150), using organic solutions with different polarity to extract the lipids followed by separation with silicic acid and ion exchange chromatography in a predetermined consecutive order. In brief, a specific mixture of organic solvents with varying polarity (chloroform and methanol) is used to extract the lipids. Following pooling and drying of the extract, mild alkaline methanolysis and dialysis degrades the ester-linkages of the glycerol-based phospholipids. Consecutive silicic acid chromatography eliminates most non-polar lipids, i.e. cholesterol-esters and free fatty acids. Succeeding ion exchange chromatography separates the acid from the non-acid (i.e. neutral) GSL. The abundant phospholipid sphingomyelin is removed from the non-acid GSL after acetylation and additional silicic acid chromatography.

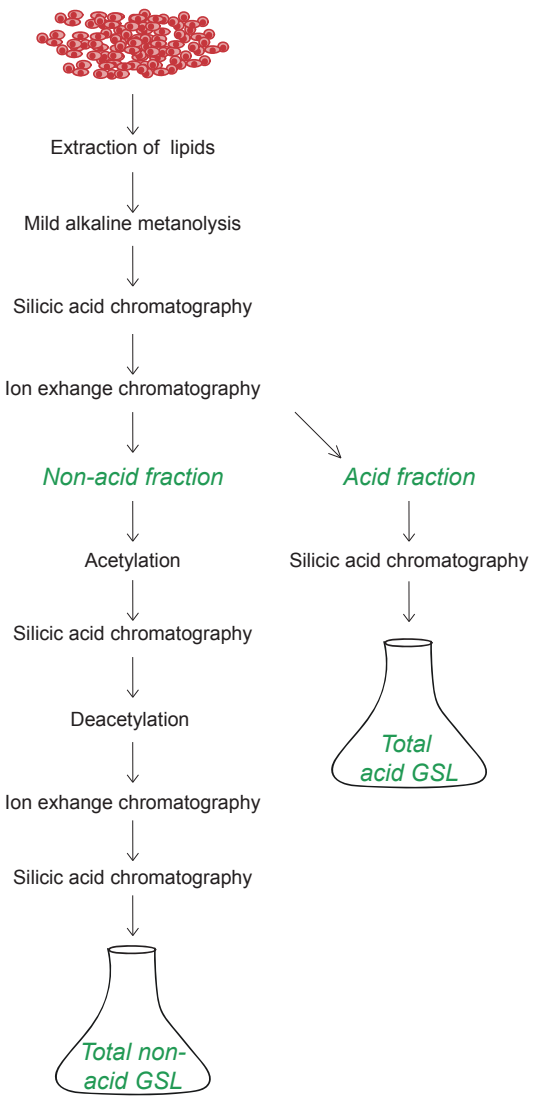

Figure 15. Schematic illustration of the protocol for isolation of acid and non-acid glycosphingolipids (GSL)*. The isolation process includes extraction of lipids with 1 to 18 carbohydrate residues developed by Karlsson (150) using different compositions of organic solvents with varying polarity (chloroform/methanol) followed by separation and purification by sialic acid and ion exchange chromatography.*

Following reinstatement of the non-acid GSL by deacetylation, further purification is accomplished by additional ion exchange and silicic acid chromatography. The latter is also applied for purification of the acid GSL. Frequent surveillance of the isolation process by thin-layer chromatography (TLC), enables validation of the consecutive preparation steps.

Mass spectrometry

Mass spectrometry (MS) is an analytical technique for measuring masses within a sample, e.g. GSL fractions, contributing to characterization of its structural composition. Mass spectrometry is a highly sensitive technique, which can analyze samples of nano (10^{-9}) and pico (10^{-12}) gram quantities. Mass spectrometry generates structural information regarding number and types of monosaccharaides, carbohydrate sequence and composition of the lipophilic part (ceramide). Analysis can be performed on a mixture of GSL or further improved by preceding liquid or gas chromatography (LS/GC). However, MS does not provide any information regarding the anomerity of the glycosidic bonds or in general, binding positions. Further characterization with proton NMR spectroscopy provides the definitive information of the oligosaccharide structure and anomerity of the glycosidic bonds, but requires substantially more material than MS and chromatographic methods (TLC/CBA). Although we had a large starting material of approximately $1x10^9$ hPSC in Papers I and II, the total amount of pure GSL obtained per cell line was only 6-10mg. Consequently, we structurally characterized the GSL with MS in combination with various binding assays such as thin-layer chromatogram binding assay including reference GSL, as well as FC, IH, IF, EM and WB described in previous sections.

In general, the MS technique entails initial ionization of the GSL and consecutive sorting of the ions based on their mass-to-charge ratio (*m/z*) by accelerating the ions in an electromagnetic field and detecting the different ions separately. Ion movement through an electromagnetic field is affected by their *m/z* ratio and consequently generates detectable separation. The mass spectrum is a graphic display of the relative abundance of ion signals against the *m/z* ratios (Figure 11). Since there are a limited number of different monosaccharide combinations consistent with a specific mass, MS analysis of intact glycans can be used to derive their compositions. Furthermore, fragmentation of the glycans enables assessment of the differences in mass between the generated fragments, providing information of the glycan sequence that can be used to distinguish oligosaccharides with the same mass by comparing the spectra with characteristic fragmentation patterns.

The MS technique we used in Papers I and II is called LC-ESI/MS, i.e. liquid chromatography electrospray ionization mass spectrometry (152). In short, GSL were separated on a graphitized carbon column (oligosaccharides from non-acid GSL) or polyamine column (acid GSL), ionized by the electrospray ionization process involving transferring of the ionic sample from solution into gas phase in an electric field. Before analyzing the non-acid GSL (Paper II), the GSL were enzymatically hydrolyzed by endoglycoceramidase II of *Rhodocuccus* sp to remove the ceramide part. Following ionization, the ions were analyzed by a LTQ linear quadrupole ion trap mass spectrometer, separating the ions in an electromagnetic field depending of their *m/z* ratio and detecting them accordingly (MS^1). Consecutively, data-dependent MS^2 (MS/MS) scans are performed to further analyze ions of a particular *m/z* ratio. Hence, data-dependent acquisition (Xcalibur software) first performs a full scan to establish the ions mass-to-charge ratio (*m/z*) and abundance of ions, followed by sequence determining MS^2 scans on ions from a subset of detected peaks in the generated mass spectrum.

The interpretation of MS data requires extensive knowledge of GSL biosynthetic pathways. The glycan sequences are manually designated by correlations of retention times and MS^2 spectra to well-characterized reference GSL, together with the computer-assisted annotation of the mass spectra of oligosaccharides with the GlycoWorkbench software (152).

4 RESULTS & DISCUSSION

This section will highlight selected results of particular interest and expand the discussion regarding these observations. The comprehensive results of the studies are presented in detail in Papers I-III.

4.1 Sialyl-lactotetra, a novel marker of undifferentiated stem cells

To facilitate research and safety enhancement of hPSC-derived products, the identification of cell surface epitopes specific for pluripotent cells are essential. Cell surface localization of such pluripotency markers enables monitoring and selection of live cells from a culture or tissue. Consequently, well-characterized biological reagents, e.g. monoclonal antibodies, detecting defined antigens can be used to identify and separate pluripotent cells from a heterogeneous population of cells or undifferentiated remnants during the differentiation process to purify cultures and reduce the risk of teratoma formation.

Glycosphingolipids are mainly present in the plasma membrane of human cells. Their exposure on the cell surface make them accessible and easily recognizable by specific antibodies. This cell surface localization in combination with the fact that GSL only have one glycan determinant, compared to glycoproteins that can have numerous, make them ideal epitopes for developing new stem cell markers. Previously, the characterization of the non-acid GSL present in hESC was performed by Barone et al. by MS, proton NMR and chromatographic methods (113). When analyzing the composition of the acid GSL of the same hESC lines (SA121 and SA181) in unique quantities in Paper I, we identified several novel hESC GSL: sialyl-globotetra, sulphatide, sulf-lactosyl, sulf-globopenta and sialyl-lactotetra. This was facilitated by structural analytic methods, including LC-ESI/MS and various immunostaining techniques using well characterized monoclonal antibodies (153, 154). Whereas for example sulphatide was mainly localized intracellularly, sialyl-lactotetra (S-Lc_4) was abundantly expressed on the cell surface of all hPSC cell lines (eight hESC, eight hiPSC according to Table 1) investigated in Papers I-III by FC, IH, EM (Figure 16, 18A). However, the structurally closely related antigen sialyl-neolactotetra was not detected in the hPSC lines investigated by MS, TLC/CBA, IH, EM or FC. Hence, no cross-reactivity apparently exists between the two antibodies although their

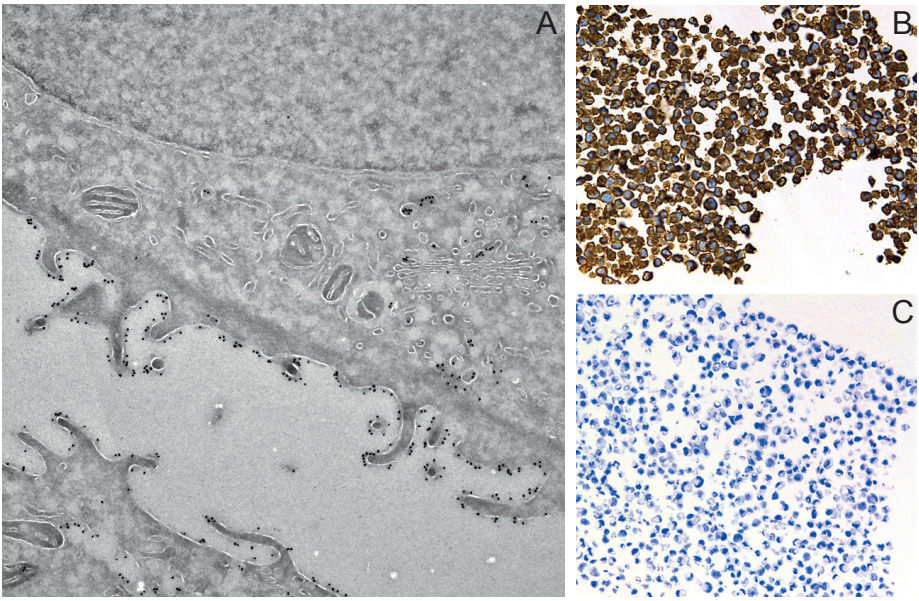

Figure 16. Sialyl-lactotetra expression, distribution and localization on the cell surface of human pluripotent stem cells (hPSC). *(**A**) Electron microscopy image showing hPSC immunostained with anti-sialyl-lactotetra and subsequently immunogold. The cell surface showed a high density of immunogold particles. Additionally, sialyl-lactotetra was present in mitochondria, vesicles and the Golgi apparatus in accordance with the biosynthesis pathways of glycoconjungates. The right column shows immunohistochemistry images visualizing the extensive staining with anti-sialyl-lactotetra antibody (**B**) and the negative reaction with anti-sialyl-neolactotetra antibody (**C**) in hPSC.*

epitopes are stereoisomers, which differ only by the binding position between the Gal and GlcNac in their carbohydrate core chains (sialyl-lactotetra, type 1 core chain/lacto series vs. sialyl-neolactotera, type 2 core chain/neolacto series). This difference may seem trivial, but the 3D structure of the epitope can be altered in such a way that it becomes unrecognizable to the antibody (155).

The expression of sialyl-lactotetra in human adult tissues is limited, despite extensive characterizations of glycoconjungates in various human tissues and organs only a few seemingly express the antigen. Sialyl-lactotetra is expressed in the frontal lobes of the immature brain, i.e. present in children up to two years of age (154). Interestingly, the expression is dynamic during the fetal and peripartum period, with almost diminished expression during the second trimester (154, 156), a transient peak around partus, followed by

declining expression until two years of age and thereafter sialyl-lactotetra expression is insignificant. However, human meconium is a rich source of sialyl-lactotetra (157) and lactotetra (Lc_4) (158). Meconium contains all extruded epithelial cells collected during the fetal period. This in combination with the finding that adult human small intestinal epithelial cells do not contain lactotetra and sialyl-lactotetra (159) indicates that these components are mainly produced, and have a potential function, during the fetal period. Interestingly, lactotetra has been found in hESC lines (113).

Besides being expressed in immature tissues, the sialyl-lactotetra structure has known association with some cancer forms such as glioma (160, 161), small cell lung cancer (162) and human embryonal carcinoma cells (163). The latter share some properties with hPSC, as discussed previously.

To further investigate the nature of the sialyl-lactotetra antigen expression in hPSC, we isolated, prepared and characterized protein extracts of hESC lines SA121 and SA181 (Paper I). Consequently, we found that both proteins as well as GSL carried sialyl-lactotetra antigens, with intensive antibody staining of the TLC/CBA and WB bands (Figure 17).

Demonstration of the abundant expression of sialyl-lactotetra on the cell surface of several hPSC cell lines, in combination with the preceding knowledge of the limited expression in adult tissue, made us identify this antigen as a potential marker of undifferentiated cells. To further explore this hypothesis we examined the alteration of expression during endodermal, mesodermal and ectodermal differentiation into hepatocyte-like cells, cardiomyocyte-like cells and neural stem cells, respectively. In comparison to pluripotency markers TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4, the anti-sialyl-lactotetra antibody showed the most intense staining in FC analyses of all investigated hPSC lines, illustrated by the right shift of the curve and 100% FITC-positivity (Figure 18A). Furthermore, the sialyl-lactotetra expression decreased rapidly during differentiation and was undetectable in the hepatocyte-like (Figure 18B) and cardiomyocyte-like cells (Figure 18C). A retaining anti-sialyl-lactotetra antibody staining (37%) with low intensity was observed in the multipotent neural stem cells (Figure 18D).

As for the other pluripotency markers investigated, TRA-1-60 showed an anticipated expression pattern, whereas the stage-specific embryonic antigens were more inconsistent. Anti-SSEA-3 staining was weak and gave divergent results between the investigated cell lines (Paper III; Figure 1) when analyzed by FC, which has been described previously (6).

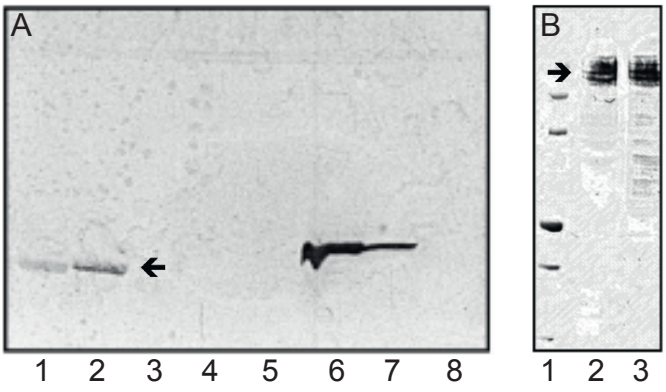

Figure 17. Sialyl-lactotetra expression in glycosphingolipids (A) and protein (B) prepared from human pluripotent stem cells (hPSC)*. (A) Thin-layer chromatogram (TLC) stained with anti-sialyl-lactotetra antibody and detected by autoradiography using a* ^{125}*I-labeled secondary antibody. Lane 1 and 2, total acid glycosphingolipids of hPSC lines SA121 and SA181, respectively; Lane 6, total acid glycosphingolipids of human hepatoma; Lane 7, total acid glycosphingolipids isolated from a lung cancer liver metastasis. (**B**) Western blot analysis of protein extracts from hPSC lines SA121 and SA181 stained with anti-sialyl-lactotetra antibody. Lane 1, molecular mass standards; Lane 2, hPSC line SA121; Lane 3, hPSC line SA181. The arrows mark the anti-sialyl-lactotetra positive bands.*

This observation can partly be explained by the mainly intracellular SSEA-3 expression demonstrated by complementary IH (Paper III; Supplemental Figure S2A). In contrast, the anti-SSEA-4 antibody was extensively expressed in the pluripotent cells (Figure 18A) and initially decreased during differentiation but was retained in about 15% of the hepatocyte-like cells (Figure 18B). However, the rebound expression of SSEA-4 from day 14 of mesodermal differentiation, resulting in 100% positivity in the cardiomyocyte-like cells (Figure 18C), is consistent with the expression found in adult cardiomyocytes (164).

The function of carbohydrate antigens such as SSEA and sialyl-lactotetra, remains to be determined. However, an indication of their significance is their seemingly tightly regulated expression during embryonic development and cell differentiation, as well as the numerous genes devoted to encoding different glycosyltransferases involved in their synthesis (108).

In the future it would be of value to further verify sialyl-lacotetra´s position as a pluripotency marker by performing functional assays, e.g. selecting sialyl- lactotetra positive cells from a heterogeneous partly differentiated cell population followed by spontaneous *in vitro* differentiation in spheres or

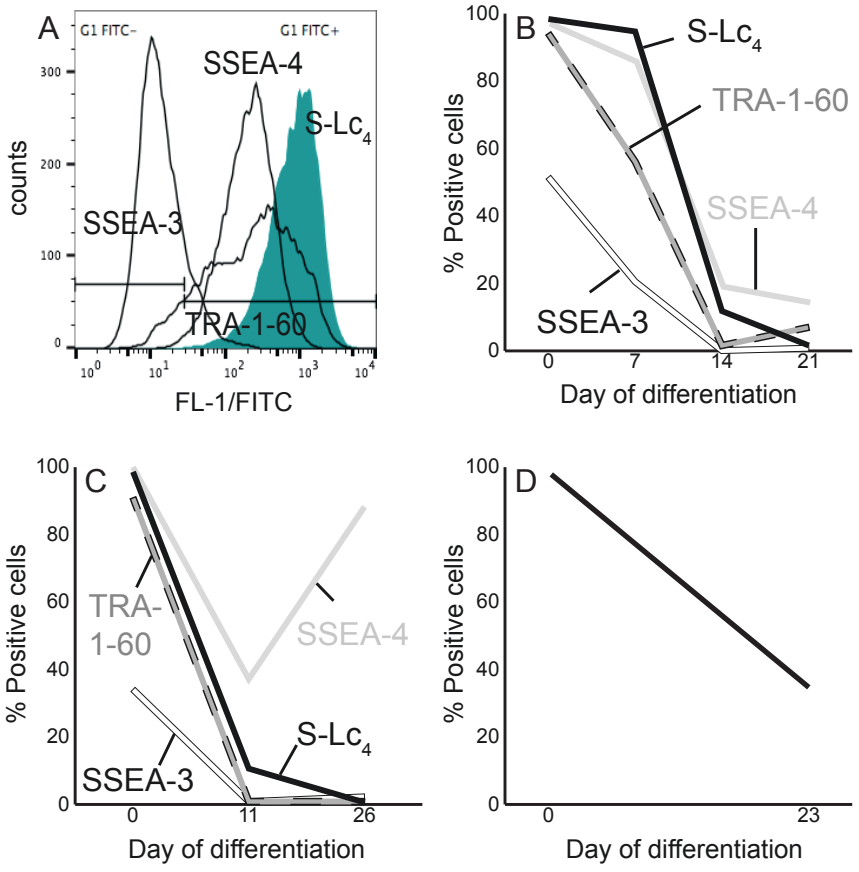

Figure 18. Expression of sialyl-lactotetra (s-Lc4) in human pluripotent stem cells and alterations during differentiation. *(**A**) The expression of sialyl-lactotetra and other alleged pluripotency markers, i.e. TRA-1-60, SSEA-3, SSEA-4, in hPSC. The horizontal bar represents the cut-off value for positive FITC reaction. (**B**) The alteration of antigen expression during endodermal differentiation into hepatocyte-like cells. (**C**) The alteration of antigen expression during mesodermal differentiation into cardiomyocyte-like cells. (**D**) The alteration of antigen expression during ectodermal differentiation into neural stem cells.*

in vivo in suitable animal models (e.g. SCID mice) to accomplish differentiation into all three embryonic germ layers or teratoma formation, respectively. However, one could argue that this already has been demonstrated since 100% of all hPSC lines we examined abundantly expressed sialyl-lactotetra and their functional pluripotency has been assessed upon derivation.

In summary, sialyl-lactotetra is a solid marker of undifferentiated cells that rapidly declines during endodermal, mesodermal and ectodermal differentiation. The IgM anti-sialyl-lactotetra antibody (154) used in our studies was developed in-house and yields consistent results in various immunostaining techniques including FC, IH, IF, CBA and EM.

4.2 Additional cell surface markers

As previously described, the availability of well characterized monoclonal antibodies detecting cell surface epitopes with high specificity is vital to the study and application of hPSC. This has stimulated the development of new antibodies fulfilling these requirements. Recently, Tang et al raised a new monoclonal antibody against the H type 1 antigen designated SSEA-5, which they identified as a novel pluripotency marker (50). In Paper III we used the same antibody to validate these observations and found expression of the H type 1/SSEA-5 in all six hPSC lines investigated (Paper III: Figure I). Furthermore, the expression rapidly declined and was in general insignificant after the first week of differentiation into hepatocyte-like and cardiomyocyte-like cells. However, the intensity of anti-H type 1/SSEA-5 antibody staining was low in hPSC when analyzed by flow cytometry and undetectable with immunohistochemistry, which may limit the usability of the antibody as a selection tool.

Besides sialyl-lactotetra, we found intense expression of Le^y in all cells of all hPSC lines investigated (Paper III: Figure 1). The Le^y expression decreased during the first 14 days of endodermal, mesodermal and ectodermal differentiation into negligible values in the cardiomyocyte-like cells, slight rebound in the hepatocyte-like cells and a significant retainment (about 70%) in the neural stem cells, although with reduced fluorescence intensity (see Papers II-III). The monoclonal anti-Le^y antibody is well characterized and structurally defined, and works well in various immunostaining techniques such as FC, IH, IC, TLC/CBA and WB (Paper II-III). Hence, this antibody could be used in combination with other antibodies to verify initiation of differentiation and identify undifferentiated cells.

Presently, the anti-Le^x antibody (P12) is often used to validate neuronal differentiation (165). However, we found a substantial subpopulation (up to 90%) of Le^x positive hPSC in the different cell lines investigated in Paper III, which complicates this application. Instead, we identified GD1a as a potential new marker for neuronal differentiation (Paper III). The anti-GD1a antibody detecting the ganglioside GD1a was negative in the pluripotent cells

(although we found small quantities of the determinant in GSL as discussed previously), whereas all neural stem cells were positively stained.

Noteworthy, O´Brien et al. recently generated seven monoclonal antibodies against defined protein epitopes on hPSC (166). Furthermore, they identified the corresponding genes, which can be assessed and analyzed, making these antibodies an interesting new contribution to the arsenal of present pluripotency markers.

4.3 Immunogenicity of hPSC and their derivatives

In recent years there has been an intense research activity in regenerative medicine, particularly in the field of stem cell technology. Presently, there are several ongoing clinical trials using hPSC-derived cells and tissues (13). However, in order to capitalize on these efforts, the safety aspects of stem cell-based therapies need to be addressed at the same pace. One area of concern is the recipient's immune response to hPSC-derived allotransplants.

The initial perception of the immune privileged state of hPSC(167) has been challenged in several studies (168, 169). However, little is still certain about hPSC expression of HLA and histo-blood group antigens. Currently, the most imminent applications of hPSC-based therapies are non-autologous transplantation of stem cell-derived cells and tissues, considering the costly strategy of more individualized approaches such as preparing hiPSC from each prospective patient. Moreover, the safety concerns raised against hiPSC-derived tissues are problematic as previously discussed and reviewed in (67, 170).

The major histocompatibility barriers, HLA and ABO histo-blood group systems, are expressed in high quantities on the surface of human cells and therefore accessible to the recipient´s adaptive immune system. Consequently, we focused mainly on cell surface expression of various carbohydrate antigens in Paper III. Our extensive profiling included three hESC and three hiPSC lines (Table 1), as well as their hepatocyte-like and cardiomyocyte-like cell derivatives.

We found quite abundant expression of HLA class I in FC and IH analysis (Figure 19). Prior studies have observed evident but low levels of HLA class I expression in hPSC (106-109). Consistently with earlier studies we did not observe any expression of HLA class II in the pluripotent cells (106-109),

nor did we note any induction during differentiation. Hence, hPSC are not professional antigen presenting cells and consequently do not possess the ability to activate T-lymphocytes via the direct allorecognition pathway facilitating acute rejection (Figure 10). Moreover, no expression of co-stimulatory molecules, CD80, CD86, ICOS and CTLA-4, necessary for successful T- lymphocyte activation was detected, which is congruous with previous findings (106).

In general, the expression of HLA class I rapidly declined during the first six days of endodermal differentiation into hepatocyte-like cells and was thereafter negligible (Figure 20A). The adult hepatocyte status of HLA class I antigens is uncertain, but ranges from variable and weak (15, 24-26) to negligible expression (18-23). No induction of HLA class II antigens was evident during differentiation, which is consistent with the conditions in healthy adult hepatocytes that do not express HLA-DQ/DR/DP (171).

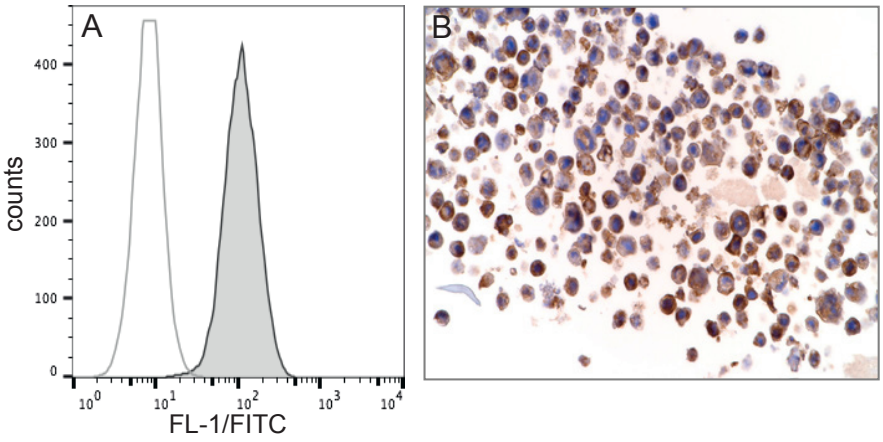

***Figure 19. HLA class I expression in human pluripotent stem cells**. **(A)** Flow cytometry analysis of anti-HLA class I antibody (filled dark grey curve) and negative control (light transparent grey curve) in hESC line SA121. **(B)** Immunohisto-chemistry staining with anti-HLA class I antibody of a majority of cells, demonstrating both intracellular and cell surface positivity.*

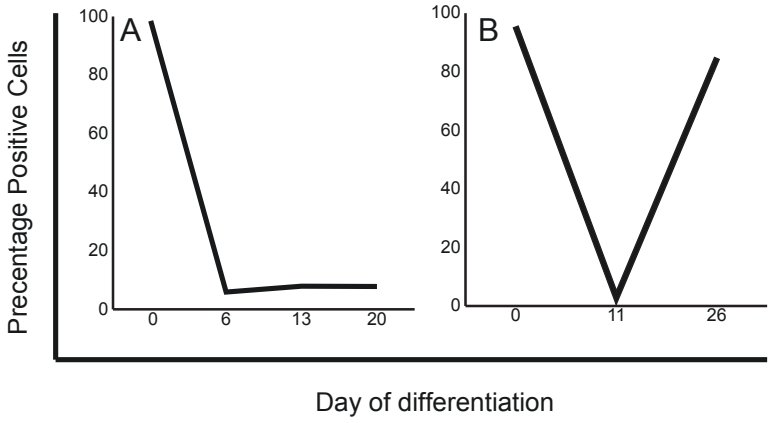

Figure 20. HLA class I expression during differentiation of hPSC line Val9 and ChiPSC22 into hepatocyte-like cells (A) and cardiomyocyte-like cells (B), respectively.

Differentiation of hPSC into cardiomyocyte-like cells resulted in an initial rapid reduction of HLA class I antigen expression, followed by a rebound expression (Figure 20B). This was unexpected since healthy cardiomyocytes lack HLA class I (172). However, HLA class I is inducible in patients with allograft rejection and myocarditis (172). Speculatively, this rebound effect could be a consequence of alterations in culturing conditions during differentiation. Consequently, identification of such factors could be used to modify the culturing and differentiation milieu to minimize the antigenicity. No induction of HLA class II expression was observed upon differentiation into cardiomyocyte-like cells, which is consistent with the adult status (172, 173).

In the clinical setting of allotransplantation it is obligate to investigate the HLA genotype of the donor and recipient in order to avoid rejection. This is often not the case in experimental studies of stem cell-derived transplants. However, descriptive characterization has generally revealed normal HLA haplotypes in hPSC lines (106, 174). The genotyping of the six hPSC lines included in Paper III was in alignment with these observations as well as a Caucasian origin of the cell lines.

The AB(O)H blood group system has generated limited attention in stem cell research. Initially it was unclear whether hPSC express blood group antigens at all (175). However, Mölne et al. characterized the ABO genotype and phenotype of nine different hESC lines and concluded that hPSC can express blood group A and B antigens (110). These results are in consistency with

our findings in Paper III. Three hPSC lines expressed blood group A antigen according to their ABO genotype, while the remaining three cell lines were of blood group O. This is a statistically expected outcome, reflecting the origin of the cell lines and the normal distribution of blood groups in the population.

Despite the coherence between the ABO genotype and phenotype, only a subpopulation of the genotype *A* hPSC lines indeed expressed the A antigen (Figure 21 A, B), although they all expressed the precursor H type 1 (Paper III, Figure 1). This result was verified in repeated flow cytometry experiments (up to n=10) and complementary cell membrane permeabilization with saponin to reveal any intracellular component contamination, as well as immunohistochemistry analysis. Hypothetically, this could be a consequence of varying activity of the blood group A_1 glycosyltransferase, depending on the maturation status of the cells.

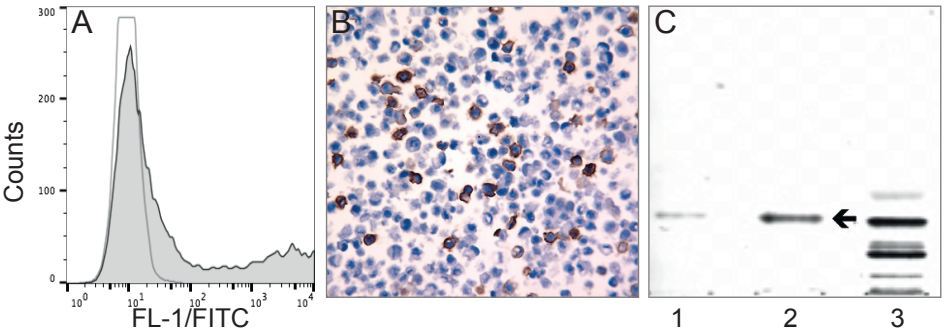

***Figure 21. Blood group A antigen expression in hPSC**. (**A**) Flow cytometry histogram of a hESC line SA181 stained with anti-A antibody. The filled grey curve represents the anti-A antibody, whereas the light grey curve is a negative control sample. Evidently, a subpopulation (35%) of the cells expressed the A antigen quite intensively, while the majority were negative. (**B**) Immunohisto-chemistry staining of hESC line SA181 with anti-A antibody. Approximately 35% of the cell demonstrated positive staining (brown precipitate). (**C**) Thin-layer chromatogram immunostained with anti-A antibody. Interestingly, there are multiple visible bands in the reference GSL fraction (lane3), compared to the single positive band (indicated by an arrow) in hPSC line SA121 (lane1) and SA181 (lane2), consisting of A type 1 hexaosylceramid. Lane 1, non-acid GSL isolated from hPSC line SA121; Lane 2, non-acid GSL of hPSC line SA181; Lane 3, non-acid GSL reference fraction from human blood group A kidney.*

Opposing this argument is the otherwise quite phenotypically and morphologically homogenous characteristics we found when exploring the cell surface tissue antigens in these hPSC lines.

Furthermore, when analyzing the isolated total neutral GSL fraction we only found a single positive band stained with the anti-A antibody (Figure 21C). This was an interesting finding, since adult cells typically express several different A antigens, as exemplified by the reference non-acid GSL isolated from a kidney from a blood group A individual, which demonstrated multiple bands. Previous biochemical studies have structurally characterized this single band to contain a blood group A GSL compound with six carbohydrate residues, i.e. A type 1 hexaosylceramide, using mass spectrometry and proton NMR spectroscopy (113).

Moreover, we did not detect any blood group A antigen expression when analyzing the protein extracts from the hPSC lines, despite extensive repetitions of the experiment and the use of three different antibodies. This indicates that the A antigen is mainly carried by lipids and not by proteins, in contrast to the expression in erythrocytes where a majority of AB(O)H antigens are protein-linked (72).

The expression of blood group A antigen decreased rapidly during mesodermal differentiation and congruent to the adult status (176), as well as previous observations (110), the cardiomyocyte-like cells lack the A antigen on their cell surface (Paper III; Figure 5). However, the alterations of the A antigen during endodermal differentiation was not as predictable or consistent between cell lines. Initially, the expression declined coherently in the two investigated blood group A hPSC lines. Surprisingly, the expression rebounded after 11 days of differentiation into hepatocyte-like cells in one of the cell lines, which is inconsistent with the status in adult hepatocytes that lack AB(O)H antigens (97, 171). However, this retainment of AB(O)H antigens during differentiation into hepatocyte-like cells has previously been described (110). Interestingly, the expression of the H type 1 precursor to A type 1, rapidly decreased from 100% positivity to insignificant expression after the first week of differentiation into both cardiomyocyte-like and hepatocyte-like cells in all investigated cell lines (Paper III: Figures 4,5).

Consequently, the results in Paper III indicate that the expression of AB(O)H antigens in the pluripotent cells and their derivatives is not a random outcome, but indeed highly reproducible and generally show quite cohesive patterns between different cell lines. These results suggest a tight control of the AB(O)H antigens expression in pluripotent cells as well as during the

differentiation process, although the developmental and physiological significance of AB(O)H antigens evidently is not essential, since individuals of the Bombay phenotype (i.e. lack AB(O)H antigens) are healthy.

In summary, hPSC abundantly express HLA class I, AB(O)H as well as other relevant histo-blood group antigens on their cell surface (Paper III), which potentially can elicit a significant immune response in the recipient following stem cell-based therapy. Our results indicate a cell line specific expression pattern of tissue and histo-blood group antigens that is not generalizable to all hPSC lines. Additionally, all cell lines expressed individual HLA genotypes depending on their origin. Thus, these factors need to be addressed and thoroughly characterized in all cell lines individually prior to allotransplantation of stem cell-derived material or before selecting suitable lines for clinical applications. Hence, following the same rigorous control and matching procedures as is protocol in the clinical routine for organ transplantation.

4.4 Are hESC and hiPSC equivalent?

A key question that remains unsolved and intensively debated in the field of stem cell research is whether hiPSC are equivalent to, and a replacement for, hESC, which was an initial hope. Are there systematic differences between hiPSC and hESC lines, and if so are such differences functionally relevant?

Convincing evidence advocating their phenotypic similarities were initially presented, including expression of pluripotency markers, ability to differentiate into all three germ layers, morphology and teratoma formation capacity (57, 177-179). However, genomic studies have shown contradicting results. Initially, studies comparing gene expression patterns highlighted their differences (62, 179), while others only revealed small and seemingly insignificant variations in chromatin structure, gene expression and DNA methylation (180, 181). The extensive genomic characterization of 20 hESC and 12 hiPSC lines by Bock et al. demonstrated global similarities, but relative stable epigenetic and transcriptional deviations that were seemingly cell line specific with overlapping variability among and between both hESC and hiPSC lines (180). On the basis of these findings, some argue that hESC and hiPSC instead should be considered overlapping heterogeneous populations (24, 182).

We addressed the question of hESC and hiPSC equivalence when exploring the composition of GSL and antigenicity in hPSC and their endodermal, mesodermal and ectodermal derivatives in Papers II-III.

Presently, only a restricted number of relevant studies have investigated the carbohydrate antigens structures and GSL in hPSC (112-114). We have recently published a review summarizing the present knowledge of GSL expression in hESC (115). Corresponding studies regarding GSL constitution in hiPSC are even more limited (114), which motivated the initiation of Paper II. Structural characterization with mass spectroscopy, chromatographic and immunostaining methods, demonstrated a high degree of resemblance between the constitution of non-acid GSL in hiPSC (Paper II) and hESC (113). However, when analyzing the acid GSL in Papers I and II some diverging results were obtained (Figure 22). Specifically, increased levels of the gangliosides GD1a and GD1b was observed in hESC compared to hiPSC, which is consistent with previous observations (114).

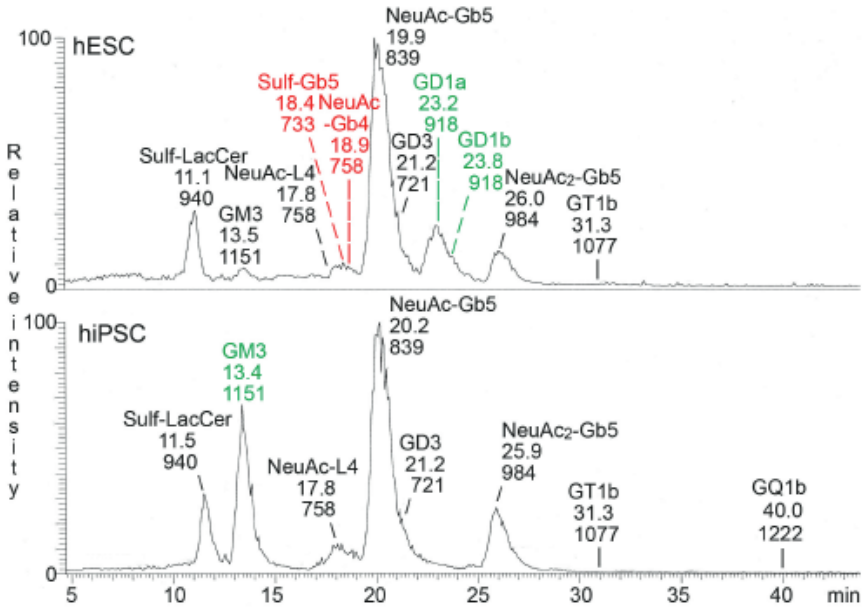

***Figure 22. LC-ESI/MS chromatograms of acid GSL in hESC (upper chromatogram) compared to hiPSC (lower chromatogram)**. Identification of the GSL was based on their retention time, molecular mass and subsequent MS^2 analysis as previously described. The sulf-globopenta (Sulf-Gb5) and sialyl-globotetra (NeuAc-Gb4) structures were identified in hESC (visualized in red color), but seemingly undetectable in hiPSC. Furthermore, the green colored peeks represent relatively higher levels of the identified GSL structure. Hence, hESC expressed higher levels of GD1a and GD1b compared to hiPSC, while the opposite relationship was true for the GM3 ganglioside.*

The reverse relationship was true for the GM3 ganglioside, where higher levels were detected in hiPSC than hESC. Furthermore, the sialyl-globotetra (NeuAc-Gb4) and sulf-globopenta (Sulf-Gb5) were only identified in hESC and consequently not found in hiPSC.

In Paper III we characterized the antigenicity of three hESC and three hiPSC lines by exploring the presence and extent of cell surface tissue antigen expression. No systematic difference in expression of HLA or histo-blood group AB(O)H and Lewis antigens was observed between the hESC and hiPSC lines in the pluripotent state or during differentiation (Paper III; Figures 1, 4, 5). Genotyping did not reveal any anomalies, nor could any distinctions be made regarding the HLA or ABO genotypes of hESC and hiPSC.

In summary, our results support the perception of hESC and hiPSC being overlapping heterogeneous *in vitro* derived populations with common features and characteristics, but of fundamentally different origin.

4.5 The misconception of Le^x and SSEA-1

The SSEA-1, Le^x, and CD15 epitopes are often synonymously used in the field of stem cell research. This is mainly a misinterpretation of the specificity of the antibodies, since the anti-SSEA-1 antibody MC480 (37) indeed recognizes the Le^x determinant, but only when present on a long type 2 core chain consisting of a minimum of seven monosaccharides (Figure 23 (40, 42, 115)). The designated anti-Le^x antibody P12 and anti-CD15 antibody C3D-1 recognize the terminal Le^x trisaccharide independently of the length of the carbohydrate core chain (Figure 23).

This distinction between SSEA-1 and Le^x antigens is important to clarify since SSEA-1 often is used as a marker for pluripotent rodent cells and for differentiation in human hPSC (6, 36). Hence, observations of Le^x expression in hPSC could be misinterpreted as initiation of differentiation.

In Paper III we investigated the expression of SSEA-1 and Le^x antigens in six hPSC lines (Paper III: Table 1, Figures 1, 6). All hPSC lines were negative for the MC480 antibody, but evidently a subpopulation of about 50% of the hPSC expressed the Le^x antigen, while the remaining cells were completely negative when analyzed by FC and IH (Figure 24 and Paper III:Table 1). Furthermore, no staining was demonstrated by the anti-CD15 (C3D-1) antibody in hESC (115).

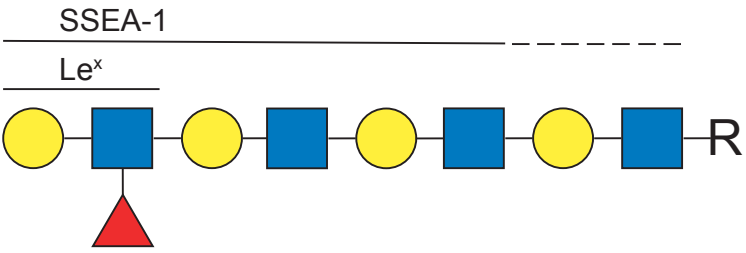

Figure 23. The structural relationship between the anti-SSEA-1 (MC480) and anti-Le^x (P12) epitopes. *As illustrated, the anti-SSEA-1 antibody recognizes a Le^x epitope when presented on a long type 2 core chain consisting of a minimum of seven monosaccharides. Thus, the dashed line is not obligate for binding of the antibody. See key to glycan structures on page vii for symbolic abbreviations. R stands for residue, i.e. lipid- or protein-linked.*

During endodermal differentiation, SSEA-1 transiently was observed with a peak of evident expression in about 10% of the cell population on day 7 (representing definitive endoderm) of differentiation into hepatocyte-like cells (Figures 24 B, C). Interestingly, the same has previously been described during differentiation of human embryonal carcinoma (EC) cells by the action of retinoic acid (183). Similarly, SSEA-1 was transiently expressed on day 7 of differentiation and thereafter declined rapidly. Additionally, only a subpopulation of cells was SSEA-1 positive, forming a group distinct from the negative population, which is in consistency with our results in hPSC. Furthermore, the transient expression of SSEA-1 has also been observed during ectodermal differentiation into neural progenitor cells (184). However, no corresponding peak or expression was noted during day 11 or 26 of mesodermal differentiation into cardiomyocyte-like cells in Paper III (day 7 was not examined).

Simultaneously with the transient expression of SSEA-1 demonstrated during differentiation into hepatocyte-like cells (day 7), the expression of Le^x reached its maximum in all investigated cell lines (Paper III, Figure 4). Consequently, the Le^x determinant on long type 2 core chains is suddenly expressed on the cell surface of definitive endoderm exclusively and thereafter rapidly declines. This pattern was observed in several different cell lines and is highly reproducible, and thus most likely not a consequence of chance and could speculatively be involved in a developmental process during embryogenesis.

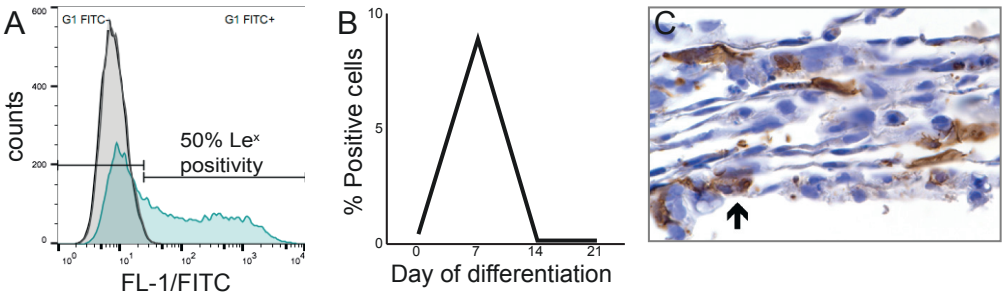

Figure 24. Expression of SSEA-1 and Le^x in human pluripotent stem cells (hPSC) and alterations during differentiation. *Flow cytometry analysis of SSEA-1 and Le^x expression in human pluripotent stem cells (**A**), alterations of SSEA-1 expression during differentiation (**B**) and staining with P12 (anti-Le^x antibody) on day 7 of endodermal differentiation into hepatocyte-like cells analyzed by immunohistochemistry (**C**). (**A**) The green filled curve illustrates the staining of hPSC with anti-Le^x antibody P12, while the filled grey curve represents the negative MC480 (anti-SSEA-1antibody) staining that overlaps the negative control (transparent black curve). The horizontal bar represents the cut-off value for positive FITC reaction. (**C**) The arrow indicates positive staining with anti-Le^x antibody.*

5 CLINICAL CONSIDERATIONS

The successful derivation of insulin-producing β-cells, cardiomyocytes, hepatocytes and neurons from human pluripotent stem cells (hPSC), opens up new therapeutic possibilities for treating patients suffering from cell, tissue or organ failure. To date, two different alternative sources of hPSC are prevailing, either isolated from *in vitro* fertilized oocytes (human embryonic stem cells, hESC) or derived through reprogramming of adult cells (human induced pluripotent stem cells, hiPSC). These separate cell types share phenotypical and functional characteristics, but have disadvantages of quite different and complex character, as previously discussed. Since the first hESC were isolated by Thomson et al. (6), a considerable amount of work has been devoted to enable clinical applications. The field took an important step when the first human trial using hPSC-derived tissue was initiated by Schwartz et al. (185, 186), with subretinal transplantation of retinal pigment epithelium derived from hESC for treatment of age-related macula degeneration. The eye is a confined and immunoprivileged site, making it attractive for studies of hPSC-based therapies. The graft was well tolerated and no adverse safety events were observed. Furthermore, they found increasing subretinal pigmentation and visual improvement. Presently, there are several on-going clinical trials with indications such as macula degeneration, diabetes and spinal cord injury (13). However, there are still many barriers to overcome before hPSC-derived therapies can become routine procedures. Transplanted cells originating from pluripotent stem cells may not function normally *in vivo*, could potentially retain tumorigenic potential, and may be rejected immunologically.

Two of the hPSC lines characterized in this thesis have been used in *in vivo* and *in vitro* studies with apparent clinical application. In a proof-of concept study of replacement therapies, hESC line Val9-derived hepatocytes were successfully engrafted and proliferated *in vivo,* rescuing hepatic function in an established mouse model of drug-induced hepatic failure (119). Additionally, the hESC Val9 line was used in the European Commission project “InnovaLiv”, where the main objective was to find new innovative strategies to generate human hepatocytes for treatment of metabolic liver diseases (131). Cell-based therapies for liver disease, i.e. hepatocyte transplantation as a therapeutic alternative to conventional liver transplantation, has previously been shown to be successful in children suffering from metabolic liver disorders, such as infantile Refsum disease (187).

Furthermore, hESC line SA121 was genetically engineered to express green fluorescent protein, allowed to spontaneously differentiate and subsequently transplanted onto a partially wounded human cornea (Bowman´s membrane with a damaged epithelium) (188). The labeled hESC-derived cells attached, proliferated and differentiated into corneal epithelial-like cells *in vitro* expressing relevant corneal epithelial markers. This demonstrates the potential of hESC-derived cells as a therapeutic alternative to the corneal necro-transplants conventionally used after e.g. chemical corneal injury.

Although promising results have been presented regarding cell based therapies and the use of hPSC-derived products (13, 23), some data call for caution. For example, successful transplantation of iPSC-derived dopaminergic neurons has been shown to improve behavior in a rat model of Parkinson´s disease (189). Additionally, evidence of successful transplantations of embryonic dopamine neurons of fetal origin to patients with Parkinson´s disease has been presented, including randomized clinical trials (190), showing transplant survival and symptomatic relief even in long-term follow ups. However, these findings have been questioned in recent years (191), and the debate is still vivid in the regenerative field. Regardless, there are lessons to be learned from these proof-of-principle studies, such as the adverse effect of dyskinesias following transplantation with e.g. hyperkinesia of predominantly choreiform movements (190, 192). This highlights the difficulties with cell-based and hPSC-based therapies; even though they can provide new neurons or tissues their function and control mechanisms can be altered and different from the native cells.

Ethical concerns surrounding the use of hESC, with political and religious inscriptions, have been evident in some parts of the world and have consequently set back the research field and limited possible clinical applications. This has however provided a strong incentive to find alternatives, which in part has resulted in the derivation of hiPSC (8) and the comprehensive development that followed.

To date, hiPSC lines have been reprogrammed from almost all adult cell types by integrating and non-integrating methods, including generation of disease-specific hiPSC (58, 59). Derivation of patient-specific hiPSC overcomes some of the substantial hurdles surrounding the clinical use of hESC, such as the ethical considerations due to their origin from *in vitro* fertilized blastocysts and the immunological challenges facing the potentially allotransplanted cells. In addition to the patient-specific hiPSC, the possibility of creating disease-specific hiPSC through reprogramming provides a unique and unlimited source of experimental materials for

studying disease characteristics, as well as *in vitro* drug development, testing and toxicology screenings (Figure 25).

In a proof-of principle study of disease-specific hiPSC derivation by Park et al., cells from patients with a variety of genetic diseases such as Parkinson´s, Huntington´s, Down´s syndrome, Gaucher disease, Becker and Duchenne muscular dystrophy were successfully reprogrammed (193). Since then numerous disease-specific hiPSC have been generated and successfully differentiated into functional adult cell types (reviewed in (13, 24)). Furthermore, the differentiated cells have been showed to be functional in animal models. For instance, Sebastiano et al. successfully reprogrammed and genetically corrected cells from patients with recessive dystrophic epidermolysis bullosa, lacking functional type VII collagen due to a mutation in the gene *COL7A1* (194). They generated patient-derived, disease-specific and gene-corrected hiPSC that subsequently were differentiated into healthy keratinocytes secreting wild-type type VII collagen and finally stratified epidermis *in vivo* in mice (see conceptual illustration in Figure 25). In the future, applications of hiPSC may include gene targeting and repair of the mutated DNA sequence, provided that the disease-causing mutation is known, as in familial Parkinson´s disease and epidermolysis bullosa.

However, low reprogramming efficiency, the postulated retainment of "epigenetic memory" of their origin (62-65), an increased tumorigenic potential due to integration of virus or reprogramming factors into the genome (61), and higher frequency of genomic anomalies found in hiPSC compared to hESC (66), may limit their therapeutic potential (67). Due to these safety-related concerns with hiPSC-based therapies, as well as the high resource consumption associated with producing individualized cell lines, mainly non-autologous hESC lines are currently used in the on-going clinical trials (13). Human pluripotent stem cell-derived cells intended for transplantation between genetically different individuals (i.e. allotransplantation) face potential rejection by the recipient's immune system.

Our studies demonstrate that all hPSC express clinically relevant tissue and histo-blood group antigens that have the potential to trigger a significant immune response. All investigated hPSC lines displayed normal HLA and ABO genotypes corresponding to their origin and phenotypical ABO blood group, respectively. The expression of HLA and histo-blood group antigens were reproducible between repetitions, but the patterns and alterations during differentiation were quite cell line specific.

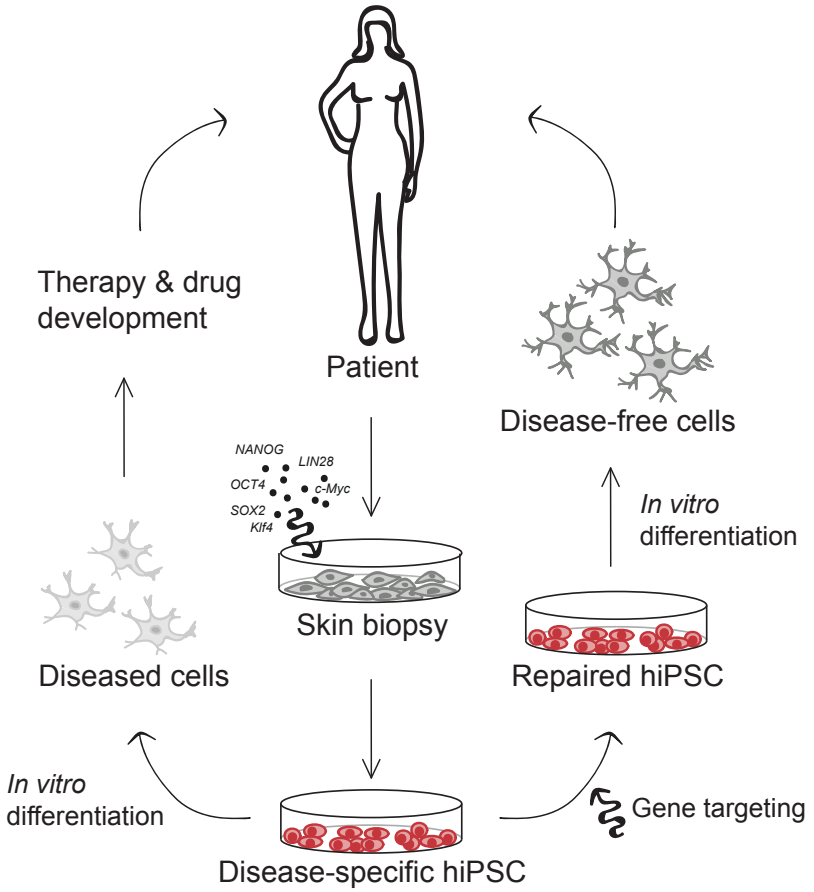

Figure 25. Potential clinical applications of human induced pluripotent stem cell (hiPSC)-derived cells*. Fibroblasts isolated by a skin biopsy from a patient with a known disease-causing mutation are reprogrammed into disease- and patient-specific hiPSC. The cells can then be differentiated into in vitro models of diseased cells providing new insights in pathophysiology as well as new concepts for therapeutic development (left), or be repaired through gene targeting of the DNA sequence in question and thereafter potentially be differentiated into disease-free cells. The healthy cells can thereafter ultimately be transplanted back to the patient using a cell replacement therapy paradigm (right).*

Consequently, the results are not generalizable and all cell lines intended for clinical application should be characterized individually. In the future, it would therefore be desirable to establish cell banks consisting of GMP hPSC-lines and hPSC-derived cells/tissues of relevant and various HLA and AB(O)H types.

In our studies we addressed yet another safety-aspect of hPSC-based applications by the identification and validation of a new marker for undifferentiated cells, sialyl-lactotetra. We have demonstrated that all pluripotent cells of several different hPSC lines express sialyl-lactotetra, in contrast to normal adult tissue that typically does not express the antigen. The sialyl-lactotetra expression declines rapidly during endodermal, mesodermal and ectodermal differentiation. Therefore sialyl-lactotetra can potentially be used to select undifferentiated cells from a heterogeneous cell population, allowing purification of the pluripotent cells in the culture or identification and exclusion of the unsuccessfully differentiated cells to reduce the risk of teratoma formation.

Prospectively, a broad exploration of the sialyl-lactotetra expression in various tumors and malignancies could conceivably enable identification of so called cancer stem cells, which are a postulated rare cell population that drives the formation and growth of tumors by their indefinite proliferative potential (15). Identification of such tumor-associated antigens available for immunotherapy and vaccine development has rendered a lot of interest in the research community as well as in the pharmaceutical industry. Interestingly, malignant transformation in cancer is often associated with changes in terminal glycan structures of cell surface antigens (195, 196). Several cancer antigens have been postulated, such as sialyl-Le^a (i.e. CA19-9, gastrointestinal and pancreatic cancer (197)), GD3 and GM2 (melanoma (198)), although the pathophysiological significance of these antigens remains to be determined.

Furthermore, Globo H and CD133 have been associated with breast cancer and putative breast cancer stem cells (199). One prerequisite for therapy desiged against targets on cancer stem cells is the absence of expression of the antigen in normal cells and tissues. In our studies all pluripotent cells intensively expressed Globo H and to a less but yet significant extent CD133. The expression of these antigens fluctuated during differentiation into hepatocyte-like cells, which expressed both CD133 and Globo H in most cell lines. Moreover, Li et al. recently postulated that CD54 (ICAM) may be involved in self-renewal and tumorigenesis of protate cancer stem cells using *in vitro* and *in vivo* assays, as well as a chemoresistant prostate cancer patient-derived xenograft model in NOD/SCID mice (200). They showed that silencing CD54, using a CD54 knocknown lentivirus, reduced tumor formation and extended the overall survival time of the tumor bearing mice. Interestingly and consistently with the Globo H and CD133 antigens, we found expression of CD54 (ICAM) in all investigated hPSC lines. In total, this contradicts the usability of Globo H, CD133 or CD54 as targets for

immunotherapy and as selection tools since there could be a potential risk for general elimination of adult stem cells in the patient. However, it provides yet another field in which hPSC can be used as a model system for evaluation and as reference material of normal differentiation.

Demonstration of Globo H and SSEA-3 in cancer stem cells (199) further motivates the exploration of GSL in hPSC as a research tool for investigation of GSL involvement in regulatory mechanisms of developmental importance. Previous studies have shown a switch in GSL core structure from the globo, lacto, neolacto series to gangliosides and their related glycosyltransferases upon hESC and hiPSC differentiation (111, 112, 114) and vice versa when comparing hiPSC to their parenteral cell type (114). These observations are in general consistent with our findings, demonstrating reduced expression of several antigens of the globo, lacto, neolacto series, e.g. SSEA 4, H-type 1, sialyl-lactotetra, and increased ganglioside expression, e.g. GD1a, during differentiation. Speculatively, the switch of GSL core structure from ganglio to globo/lacto/neolacto series in hPSC may contribute to the maintenance of their undifferentiated state or endless self-renewing capacity. The latter being an ability they share with malignant cells and cancer stem cells (15).

The histo-blood group related Le^y antigen has been associated with various malignancies (201, 202). Our results demonstrate that all pluripotent cells express Le^y. The expression of Le^y could potentially be associated with the self-renewing capacity of both hPSC and cancer cells. Several signaling pathways, e.g. Notch and Wnt, involved in regulation of self-renewal during normal stem cell development are indeed associated with malignant transformation and oncogenesis (15). Interestingly, c-Myc which is one of the transcription factors used during reprogramming of adult cells to hiPSC, speculatively accounts for the similarities in transcription programs demonstrated between hESC and cancer cells (203). Furthermore, animal studies have revealed a connection between iPSC derivation and tumorigenesis, demonstrating that the same transcription factors used for reprogramming to pluripotency drive tumor initiation *in vivo* (204, 205).

In summary, hPSC-based model systems enable unique possibilities to study and increase the knowledge of normal and pathological human development, which can generate new clinical prevention and therapeutic strategies. Furthermore, the potential of hPSC-based therapies are practically endless and will hopefully become a clinical reality for the next generation. However, substantial experimental and clinical research is needed before this can become a reality.

ACKNOWLEDGEMENTS

I wish to express my sincerest gratitude to all my co-authors, colleagues, friends and family who have contributed to the completion of this thesis. In particular,

University of Gothenburg, Sahlgrenska Academy; represented by **Peter Naredi**, Head of the Institute of Clinical Sciences and **Eva Angenete** Head of the Department of Surgery, for giving me the opportunity to complete my PhD, as well as my Master of Science degrees in Medicine and Pharmaceutical Bioscience.

Michael Breimer, my main supervisor, for generously sharing your profound knowledge of glycobiology and basic science, excellent scientific guidance and for encouraging me to thrive to become an independent scientist.

Susann Teneberg & **Johan Mölne**, my co-supervisors, for your scientific contributions, executive abilities, invaluable engagement and support.

Angela Barone, my scientific co-worker, for our common devotion to sialyl-lactotetra, all magnificent electron microscopy images, your contagious enthusiasm and big heart.

Lennart Rydberg, for introducing me to transfusion and transplantation medicine, as well as giving me access to the laboratory facilities and competent staff at the tissue-typing unit at the Sahlgrenska University Hospital. Especially, **Mats Bemark**, for valuable guidance and assistance with interpretation of flow cytometry data.

The involved staff at Takara Bio Europe (former Cellartis AB) for providing the stem cells used in our studies. Especially, **Anders Aspegren**, for our fruitful collaborations and rewarding discussions.

Christel Jansson, for excellent administrative support and friendship.

My former and present Heads of Departments at the Sahlgrenska University Hospital, **Ulf Angerås**, **Anders Hyltander**, **Christina Svärd**, **Hans Lönroth**, **Claes Jönsson, Erik Johnsson** and **Anna Elander**, for encouraging and supporting me to pursue both my clinical and scientific ambitions.

All my **friends** and **colleagues** at the surgical clinics at the Sahlgrenska University Hospital (Östra and Sahlgrenska), for generously sharing your surgical skills, knowledge and wisdom. Especially, **Anders Thornell**, my invaluable friend and ex-fellow intern, resident and PhD student, for filling these sometimes challenging and intensive years with joy and laughter.

All my beloved **friends** and **family**, thank you for all your patience, indulgence with my absence, encouragement and support. Especially, **Lotta**, **Per**, **Anders** and **Petter Hallberger,** who I truly appreciate and value as my extended family. **Britt** & **Bernt Faijerson**, my in-laws, for your generous and including ways and for the love you show for our family.

Björn Säljö, for being a true big brother; loving, protective and rock solid.

Lena & **Roger Säljö**, my parents, for giving my endless possibilities in life, freedom to prosper, the courage to pursue my dreams and yet a humble and nuanced view of our privileged lives.

Olle & **Lisa Säljö**, our gorgeous children, for keeping me focused, grounded and for sharing all your wondrous insights on life.

Jonas Faijerson Säljö, my husband, for your endless love and support, brilliant mind and beautiful heart.

This thesis was supported by grants from the Gothenburg Medical Society, Professor Lars-Erik Gelin Memorial Foundation for Transplant Research and the Swedish Medical Research Council, the Swedish Cancer Foundation and governmental grants to the Sahlgrenska University Hospital through the ALF agreement.

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