

Finnish Red Cross Blood Service  
and  
Faculty of Biological and Environmental Sciences,  
University of Helsinki,  
Finland

**GLYCAN BINDING PROTEINS IN THERAPEUTIC  
MESENCHYMAL STEM CELL RESEARCH**

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ACADEMIC DISSERTATION

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“It is simply this: do not tire, never lose interest, never grow indifferent  
—lose your invaluable curiosity and you let yourself die.  
It's as simple as that.”

-Tove Jansson

# CONTENTS

ORIGINAL PUBLICATIONS .....	7
ABBREVIATIONS .....	8
ABSTRACT .....	10
REVIEW OF THE LITERATURE .....	11
1 MESENCHYMAL STEM CELLS .....	11
1.1 Stem cell classes .....	11
1.1.1 Human pluripotent stem cells .....	11
1.1.2 Adult multipotent stem cells .....	12
1.2 Characteristics of mesenchymal stem cells .....	13
1.2.1 History .....	13
1.2.2 Defined characteristics.....	14
1.2.3 Heterogeneity.....	14
1.2.4 Plasticity .....	15
1.3 Mesenchymal stem cell therapy.....	16
1.3.1 Mesenchymal stem cells in tissue engineering.....	16
1.3.2 Immunomodulatory properties of mesenchymal stem cells .....	17
1.3.3 Mesenchymal stem cells in clinical trials .....	18
2 GLYCOBIOLOGY .....	19
2.1 Glycans on the cell surface .....	19
2.1.1 Glycocalyx .....	19
2.1.2 Glycan structures .....	20
2.2 Cellular glycobiology .....	23
2.2.1 Blood group antigens on red cells .....	23
2.2.2 Selectins in leukocyte rolling.....	24

2.2.3	Sialyl Lewis x in fertilization .....	25
2.2.4	Cell surface glycans in microbial binding.....	25
2.2.5	Differential glycosylation in cancer malignancy .....	26
2.3	Glycan binding proteins .....	27
2.3.1	Lectins .....	27
2.3.2	Glycan specific antibodies .....	29
2.3.2.1	Antibody isotypes .....	29
2.3.2.2	Availability .....	30
2.3.2.3	Production.....	30
2.3.2.4	Specificity .....	31
2.4	Use of glycan binding proteins.....	31
2.4.1	Glycan binding proteins as reagents in the study of glycans .....	31
2.4.2	Glycan binding proteins in diagnostics and therapy .....	32
2.4.3	Challenges related to glycan binding proteins .....	33
3	GLYCANS IN STEM CELL BIOLOGY.....	35
3.1	The glycan markers of stem cells .....	35
3.1.1	Glycome profile .....	35
3.1.2	Surface antigens .....	35
3.1.3	Glycosyltransferases .....	37
3.2	Role of glycans in stem cell cultures.....	38
3.2.1	Lectin as embryonal stem cell culture matrix .....	38
3.2.2	Non-human glycans in cultured stem cells .....	39
3.2.3	Metabolic glycoengineering.....	40
3.3	Glycosylation in stem cell biodistribution .....	40
3.3.1	Glycans in hematopoietic stem cell homing .....	40
3.3.2	Glycans in mesenchymal stem cell homing .....	41

SUMMARY OF THE STUDY .....	43
4 AIMS OF THE STUDY .....	43
5 MATERIALS AND METHODS .....	44
5.1 Methods .....	44
5.2 Ethics .....	45
6 RESULTS.....	46
6.1 Characterization of mesenchymal stem cell glycome (I, II) .....	46
6.2 The i antigen on the surface of mesenchymal stem cells (I, II) .....	48
6.3 Production of glycan binding proteins (III, IV) .....	49
6.4 Epitope determination of glycan binding proteins (II, III, IV) .....	51
6.4.1 Enzymatic and chemical cell surface modification (II, II, IV) .....	51
6.4.2 Competition binding assay (III).....	52
6.4.3 Glycan array (III, IV).....	52
6.4.4 Sequence analysis and comparison (III) .....	52
6.4.5 The occurrence of the epitope (I, II, III, IV).....	53
DISCUSSION.....	55
Glycans are potential biological biomarkers to be used in stem cell characterization and therapy.....	55
Stem cell surface glycans are characteristic to a cell type .....	56
Linear poly-N-acetyllactosamine (i antigen) is a marker for mesenchymal stem cells .....	57
Mesenchymal stem cell surface glycans introduce other alternative markers .....	58
Future prospects in stem cell glycomics.....	59
ACKNOWLEDGEMENTS.....	60
REFERENCES .....	62

# ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I- IV:

- I Heiskanen A, Hirvonen T, Salo H, Impola U, Olonen A, Laitinen A, Tiitinen S, Natunen S, Aitio O, Miller-Podraza H, Wuhrer M, Deelder AM, Natunen J, Laine J, Lehenkari P, Saarinen J, Satomaa T, Valmu L. (2009) Glycomics of bone marrow-derived mesenchymal stem cells can be used to evaluate their cellular differentiation stage. *Glycoconj J*. 2009 Apr;26(3):367-84.
- II Hirvonen T\*, Suila H\*, Kotovuori A, Ritamo I, Heiskanen A, Sistonen P, Anderson H, Satomaa T, Saarinen J, Tiitinen S, Rabinä J, Laitinen S, Natunen S, Valmu L. (2012) The i blood group antigen as a marker for umbilical cord blood-derived mesenchymal stem cells. *Stem Cells Dev*. 2012 Feb 10;21(3):455-64. (\* equal contribution)
- III Hirvonen T, Suila H, Tiitinen S, Natunen S, Laukkanen ML, Kotovuori A, Reinman M, Satomaa T, Alfthan K, Laitinen S, Takkinen K, Rabinä J, Valmu L. (2013) Production of a recombinant antibody specific for i blood group antigen, a mesenchymal stem cell marker. *Biores Open Access*. 2013 Oct;2(5):336-45.
- IV Hirvonen T, Henno H, Tiitinen S, Lampinen M, Laitinen S, Rabinä J, Höyhty M, and Valmu L. Production and characterization of monoclonal antibodies against glycans on mesenchymal stem cell surface. *manuscript*

\* These authors contributed equally to the study.

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# ABBREVIATIONS

Ab	Antibody
Asn	Asparagine
BM	Bone marrow
BM-MSC	Bone marrow derived mesenchymal stem/stromal cell
CFG	Consortium for Functional Glycomics
CRD	Carbohydrate recognition domain
DELFLIA	Dissociation-enhanced lanthanide fluorescent immunoassay
DSA	<i>Datura stramonium</i> agglutinin
ECA	<i>Erythrina cristacalli</i> agglutinin
e.g.	<i>exempli gratia</i> , for example
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cell
FCS	Fetal calf serum
FRC	Finnish Red Cross
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	N-acetylgalactosamine
GBP	Glycan binding protein
Glc	Glucose
GlcNAc	N-acetylglucosamine
GSL	Glycosphingolipid
GvHD	Graft-versus-host disease
HCELL	Hematopoietic cell E-/L-selectin ligand
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSPG	Heparan sulfate proteoglycan
Ig	Immunoglobulin
iPS cell	Induced pluripotent stem cell
ISCT	International Society of Cellular Therapy
IVF	<i>In vitro</i> fertilization
LacNAc	N-acetylglactosamine
LEA	<i>Lycopersicon esculentum</i> agglutinin
Man	Mannose
MSC	Mesenchymal stem/stromal cell
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NK cell	Natural killer cell
NMR	Nuclear magnetic resonance
PSA-NCAM	Polysialylated neural cell adhesion molecule



PSGL-1	P-selectin glycoprotein ligand-1
PWA	<i>Phytolacca Americana</i> agglutinin
scFv	Single chain variable fragment
sLex	Sialyl Lewis x
SSEA	Stage-specific embryonic antigen
STA	<i>Solanum tuberosum</i> agglutinin
Ser	Serine
Thr	Threonine
Tra	Tumor-rejection antigen
UCB	Umbilical cord blood
UCB-MSC	Umbilical cord blood derived mesenchymal stem/stromal cell

## **ABSTRACT**

Mesenchymal stem/stromal cells (MSCs) are multipotent adult stem cells that hold enormous therapeutic potential. They are currently in a focus of intense clinical and scientific investigation. MSCs are a promising cell type for various applications in the field of tissue engineering due to their multi-lineage differentiation capacity. Furthermore, one of their most interesting characteristics is that they possess immunomodulatory properties making these cells an attractive candidate for therapy of several immune-mediated disorders. MSCs are of nonembryonic origin and thus provide a less controversial and technically more feasible alternative for ESCs in future therapeutic applications.

Due to their location on the cell surface, glycans are ideal molecules for identification, purification, and characterization of cells for therapeutic purposes. Methods to reliably and proficiently determine both the change in the presence of a specific glycan structures and the changes in the glycome profile of a cell, are needed. Glycan binding proteins in general serve as diagnostic tools in medical and scientific laboratories. High affinity and exquisite specificity are important factors for their successful use.

The aim of this study was to characterize the glycans on the surface of MSCs in order to find novel MSC specific glycan markers. Further goal was to develop antibodies specific for MSC surface glycans, including the novel MSC marker.

As described in the original publications of this study, we first characterized the glycome of MSCs and discovered that certain specific glycan epitopes are present only in MSCs, and not in cells differentiated from them. These epitopes include i antigen, which was further characterized to be a marker for umbilical cord blood derived MSCs. An antibody against the i antigen was generated using recombinant technology. Antibodies recognizing MSC surface glycans were also generated by utilizing hybridoma technology, using whole MSCs in the immunization.

Taken together, these studies provide information of the changes in the glycome profile during MSC differentiation and describe a novel MSC marker. In these studies, we used two different methods to generate anti-glycan antibodies and emphasize the importance of thorough characterization of the binding properties of GBPs. The information of the characteristic glycosylation features of MSCs, and specific markers especially, can be used to isolate and characterize desired, therapeutically advantageous cell populations for distinct applications. Development of better glycan binding proteins will advance the field of cellular therapy and also the glycobiological research in general.

# REVIEW OF THE LITERATURE

## 1 MESENCHYMAL STEM CELLS

### 1.1 Stem cell classes

Stem cells are undifferentiated cells that are present throughout life, from embryo to adult. They are defined by their capacity for self-renewal, high differentiation potential, and their ability to differentiate into different progenitor and mature cell types. The classification of stem cells is based on their development potential. The most versatile cell type is the totipotent stem cell, e.g. the fertilized oocyte and cells in the early embryo, capable of giving rise to all embryonic and extraembryonic cell types. These cells are able to specialize and form the blastocyst. Embryonic stem cells from the inner cell mass of this blastocyst are called pluripotent, meaning they have the potential to develop into all different cell types found in an embryonic and adult organism, excluding extraembryonic organs, such as placenta and umbilical cord. Multipotent stem cells, such as mesenchymal stem/stromal cells (MSCs) and hematopoietic stem cells (HSCs) are able to differentiate into multiple, but limited cell types. Cells able to give rise to few or one specific cell lineages are called tissue-specific progenitor cells (Alison et al. 2002).

#### 1.1.1 Human pluripotent stem cells

Pluripotent human embryonic stem cells (ESCs) are, in theory, the most versatile cell type for application in regenerative medicine. ESCs can be grown from the inner cell mass of human embryos produced by *in vitro* fertilization (IVF). The first immortal human ESC lines were produced in 1998 (Thomson et al. 1998). ESCs have been hailed as a promising source of therapy for a wide variety of human diseases, including Parkinson's disease, diabetes mellitus and Alzheimer's disease. The ethical issues surrounding the use of IVF embryos, the lack of understanding how to specifically regulate ESC differentiation, and the widely reported tumorigenicity associated with ESC experimental models have driven researchers to use adult stem cells that lack these drawbacks (Salem and Thiemermann 2010).

Few years ago researchers managed to reprogram human somatic cells into pluripotent state by retroviral transduction of just four genes of regulatory transcription factors; Oct4, Sox2, Klf4 and c-Myc (Takahashi et al. 2007, Park et al. 2008). These cells are called induced pluripotent stem (iPS) cells. iPS cells can be used to generate patient- and disease-specific pluripotent stem cells, but there are number of challenges to overcome if iPS cells were to be applicable in regenerative medicine. Major concerns of iPS cells are caused by the low efficiency of iPS cell generation without genetic alterations, the possibility of tumour formation *in vivo*,

the random integration of retroviral-based delivery vectors into the genome, and unregulated growth of the remaining cells that are partially reprogrammed and refractory to differentiation (Madonna 2012). The development of iPS cells has not replaced the use of human ESCs but has offered additional insights into understanding disease mechanisms and a suitable tool for personalized medicine such as drug screening and toxicology (Shtrichman et al. 2013).

### **1.1.2 Adult multipotent stem cells**

HSCs and MSCs are non-embryonic, adult stem cells and thus provide a less controversial and technically more feasible alternative for embryonic stem cells in future therapy applications (Moore et al. 2006, Pessina and Gribaldo 2006).

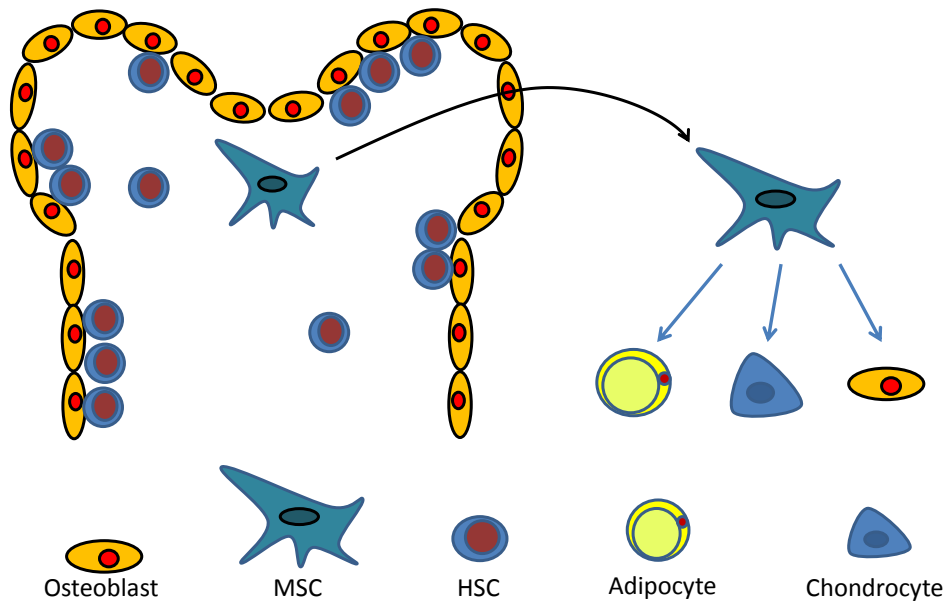
HSCs can differentiate to produce all mature blood cell types in the body. Human blood contains a large variety of differentiated cells with a limited half-life, therefore new blood cells need to be provided continuously by multipotent HSCs. The primary source of HSCs in the adult is the bone marrow, but HSCs from umbilical cord blood and peripheral blood are also clinically used (Arcese et al. 1998, Ng et al. 2004). It is known that a small number of HSCs can expand to generate a very large number of daughter HSCs as well as progenitor cells and differentiated blood cells. This phenomenon is utilized in bone marrow transplantation, where a small number of HSCs reconstitute the hematopoietic system after chemotherapy or irradiation used to destroy the patients own bone marrow. Today, HSC transplant is the only stem cell therapy widely used in clinical practice to treat patients with hematological malignancies (Helmy et al. 2010).

MSCs are an excellent cell type for therapeutic applications, since they lack the ethical considerations of ESCs and the safety concerns of iPS cells. MSCs are multipotent cells originally isolated from the bone marrow and subsequently also identified in various other adult and fetal tissues (Kern et al. 2006, Campagnoli et al. 2001, Tsai et al. 2004). MSCs are currently in a focus of intense clinical and scientific investigation. Due to their multi-lineage differentiation capacity, they are a promising cell type for various applications in the field of tissue engineering (Pittenger et al. 1999). MSCs have also been shown to be capable of improving engraftment of hematopoietic stem cells after allogeneic transplantation (Koc et al. 2000, Dazzi et al. 2006). One of the most interesting features of MSCs is that they possess immunomodulatory properties and that makes these cells an attractive candidate for therapy of several immune-mediated disorders (English et al. 2010, Yi and Song 2012).

## **1.2 Characteristics of mesenchymal stem cells**

### **1.2.1 History**

MSCs were first identified from the bone marrow in the 1960s by McCulloch and Till, who first revealed the clonal nature of these cells (Siminovitch et al. 1963, Becker et al. 1963). MSCs were further investigated in the 1970s by Friedenstein and colleagues, who demonstrated their capacity for self-renewal and multi-lineage differentiation and named the cells colony-forming unit fibroblasts (Friedenstein et al. 1974, Friedenstein et al. 1987). The term mesenchymal stem cell, cell capable to differentiate into all cells of mesodermal lineage, was coined by Caplan in 1991 (Caplan 1991). Caplan's group was also the first one to isolate these cells from the human bone marrow (Haynesworth et al. 1992). Since then, MSCs have been isolated from number of other sources, including umbilical cord blood (UCB), adipose tissue, liver, and amniotic fluid (Kern et al 2006, Campagnoli et al. 2001, Tsai et al. 2004). The physiological role of MSCs in the bone marrow is thought to be the maintenance of the HSC microenvironment and the control of their quiescence or proliferation, differentiation and recruitment (Friedenstein et al. 1974, Dazzi et al. 2006, Uccelli et al. 2008). Nowadays, International Society of Cellular Therapy (ISCT) recommends the term multipotent mesenchymal stromal cell instead of mesenchymal stem cell (maintaining the acronym MSC) (Horwitz et al. 2005), to point out that these cells are a heterogenous population of cells, not all of them necessarily having self-renewal capacity required for stem cells. Both terms are widely used in the literature.



**Figure 1** MSCs reside within the stromal compartment of bone marrow where they play a role in providing the stromal support system for HSCs. MSCs represent a very small fraction, 0.001–0.01% of the total population of nucleated cells in marrow. However, they can be isolated and expanded with high efficiency, and induced to differentiate to osteoblasts, chondrocytes, and adipocytes under defined culture conditions (Barry and Murphy 2004). Modified from Uccelli et al. 2008 and Dazzi et al. 2006).

### 1.2.2 Defined characteristics

Biological and clinical interest in MSC has risen dramatically over last two decades, but the defining characteristics of MSC have been inconsistent among investigators. Many laboratories have developed methods to isolate MSCs. They have been isolated from many different sources and expanded in different culture conditions. Variations on methodologies and tissue sources result inevitably to a question whether the resulting cells are sufficiently similar to be compared for biological properties, experimental outcomes, and therapy applications. A particular challenge has been the absence of a specific marker to define MSCs. In 2006 the ISCT defined minimal criteria for MSCs (Dominici et al. 2006). According to these criteria MSCs have to be plastic adherent, and express surface antigens CD105, CD73, and CD90. MSCs have to lack the expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLAII (predominantly markers of hematopoietic cells), to exclude cells most likely to be found in MSC cultures. To meet the criteria, MSCs also have to be able to differentiate to osteoblasts, adipocytes, and chondroblasts.

### 1.2.3 Heterogeneity

Most of the research of MSCs is focused on bone-marrow derived MSCs (BM-MSCs) and these are also overrepresented in clinical trials (Helmy et al. 2010, English et al. 2010). However, as other attractive sources for MSCs exist, these

should be thoroughly considered for their slightly different features and the availability of their source (Lv et al. 2012, Akimoto et al. 2013, Strioga et al. 2012). A source of MSCs could be selected according to the intended application. Based on their availability, umbilical cord blood and adipose tissue have become promising sources of MSCs (Kern et al. 2006).

Even if MSCs isolated from different sources meet all the criteria required, the cells are not uniform (Kern et al. 2006). MSCs obtained from different tissues have been reported to have differences in gene expression, diverse differentiation potential, proliferation capacity, and differences in surface antigens other than stated in the requirements of minimal criteria (Kern et al. 2006, Lu et al. 2006, Alviano et al. 2007). Some of the differences may represent specific features of MSCs from different origins and some may be related to different isolation and culture protocols (Strioga et al. 2012).

The culture expanded MSC population may be heterogeneous and represent several generations of different types of mesenchymal cell progeny with differing proliferation and differentiation potentials (Reiser et al. 2005). Parameters such as plating density, number of passages, and especially culture medium may have profound effects to the cells (Sotiropoulou et al. 2006, Bieback et al. 2009). The cell culture conditions may influence the properties, especially immunomodulatory effects of MSCs even more than the MSC source (Helmy et al. 2010). Cells that are aimed at therapy applications, should be cultivated in a medium free of any animal derived constituents. These could result in the production of animal derived glycans, such as N-glycolylneuraminic acid (Neu5Gc), on the cell surface, potentially causing problems when the cells are given to a patient (Varki 2001, Heiskanen et al. 2007, Tangvoranuntakul et al. 2003).

#### **1.2.4 Plasticity**

The ability of MSCs to differentiate to other cell lineages than cells from mesodermal origin is called transdifferentiation, or plasticity. MSCs, being of mesodermal origin, have been reported to differentiate *in vitro* into endoderm and ectoderm lineages, including neural cells (Sanchez-Ramos et al. 2000, Krampera et al. 2007), hepatocytes (Schwartz et al. 2002), and epithelial cells (Spees et al 2003). Whether the plasticity is a relevant issue *in vivo*, is still controversial and differing opinions are found in the literature. Also, transdifferentiation may just be the result of prolonged culture expansion under specific culture conditions (Nauta and Fibbe 2007, Fernandez Vallone et al. 2013).

Plasticity was initially hailed as a promising property widely applicable therapeutically. More recent findings suggest that the ability of MSCs to alter the tissue microenvironment via secretion of soluble factors may contribute to tissue repair more significantly than their capacity for transdifferentiation (Phinney and Prockop 2007).

## 1.3 Mesenchymal stem cell therapy

Stem cells hold enormous therapeutic potential in various medical applications. Regenerative medicine is an emerging interdisciplinary field of research and clinical application. It is focused on the replacement or regeneration of human cells, tissues or organs, to restore or establish normal function (Mason and Dunnill 2008). The differentiation potential and immunomodulatory functions of MSCs have generated wide interest in regenerative medicine and MSCs have been hailed to revolutionize the field. MSCs might become an efficacious tool to treat several degenerative disorders, in particular those requiring the repair of damaged tissues together with an anti-inflammatory effect (English et al. 2010, Bernardo et al. 2012). The mechanisms through which MSCs exert their therapeutic potential rely on the ability of MSCs to secrete soluble factors capable of stimulating survival and recovery of injured cells, the capacity to home to damaged tissue, and to produce paracrine factors with anti-inflammatory properties, resulting in functional recovery of the damaged tissue (Bernardo et al. 2012). In terms of the clinical applications of MSCs, they are being tested mainly in tissue regeneration, treatment of immune diseases, and enhancement of HSC engraftment (Helmy et al. 2010).

### 1.3.1 Mesenchymal stem cells in tissue engineering

A part of regenerative medicine is the use of MSCs in tissue engineering. Tissue engineering takes advantage of the combined use of cultured living cells and three-dimensional scaffolds to deliver vital cells to the damaged site of the patient. MSCs have been proven effective in the treatment of bone and cartilage defects in a number of animal models. These include repairing bone defects of dogs with implants loaded with autologous MSCs (Bruder et al. 1998), skull defects of rabbits with scaffolds containing osteoblasts and BM-MSCs (Schantz et al. 2003), and bone-tendon junction repair of rats, where MSC treatment was shown to produce better organ regeneration than chondrocyte treatment (Nourissat et al. 2010). MSCs have also been used in numerous experimental and clinical studies to treat bone and cartilage defects in humans. They have shown to be efficacious in the treatment of large bone defects (Quarto et al. 2001) and defects of articular cartilage (the smooth, white tissue that covers the ends of bones where they come together to form joints) (Wakitani et al. 2004, Haleem et al. 2010). Adipose derived MSC (ASC) products have been used in the treatment of bone defects such as maxillary reconstruction (Mesimäki et al. 2009). MSCs have also been shown to ameliorate Osteogenesis Imperfecta, a severe genetic disease characterized by production of defective type I collagen, causing fractures and retarded bone growth. The therapeutic effect was demonstrated by showing that after allogeneic intravenous bone marrow transplantation BM-MSCs can engraft in humans and generate donor-derived osteoblasts that function sufficiently well for a period of time and attenuate biochemical, structural and clinical abnormalities associated with Osteogenesis Imperfecta (Horwitz et al. 1999, Horwitz et al. 2001).



### 1.3.2 Immunomodulatory properties of mesenchymal stem cells

In addition to use in tissue engineering, MSCs have generated great interest for their ability to display immunomodulatory effects. They may play specific roles in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, as well as tumor evasion.

The anti-inflammatory effects of MSCs on many cell types on both innate and adaptive immune systems have been demonstrated to be broad (reviewed in Nauta and Fibbe 2007, Uccelli et al. 2008, and English et al. 2010). The first indications for the immunosuppressive nature of MSCs were obtained from the studies showing that MSCs were able to strongly suppress T cell activation and proliferation *in vitro* (Di Nicola et al. 2002, Bartholomew et al. 2002). In addition, MSCs have been shown to modulate immune response through the induction of regulatory T cells (Treg) (Selmani et al. 2008), a specialized subpopulation of T cells that suppress activation of the immune system and thereby help to maintain homeostasis and tolerance to self antigens. MSCs have also been shown to modulate functions of antibody-producing B cells by inhibiting their proliferation, differentiation, and antibody production *in vitro* (Corcione et al. 2006). MSCs suppress the differentiation of dendritic cells, resulting in the formation of tolerogenic immature cells that do not function as antigen presenting cells to T cells (Jiang et al. 2005, Nauta et al. 2006). MSCs inhibit proliferation and cytotoxicity of natural killer (NK) cells (Spaggiari et al. 2006). NK cells identify and kill allogeneic cells and their involvement can have a significant impact on the outcome of organ transplantation.

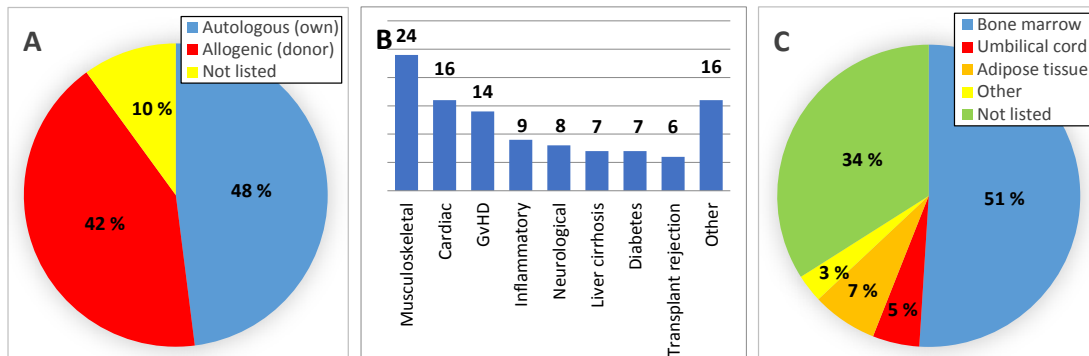
Many immunosuppressive mechanisms of MSCs have been shown to be mediated by soluble factors collaborating with contact-dependent mechanisms of cell surface receptors. MSC-mediated immunoregulation is a multilateral system that is mediated by several either constantly expressed or induced molecules (Uccelli et al. 2008). Evidence is now emerging that the local microenvironment is key in the activation (or 'licencing') of MSCs to become immunosuppressive. MSCs probably are not spontaneously immunosuppressive, but require activation by inflammatory cytokines to exert their immunosuppressive effects (English 2013, Krampera 2011). Further *in vivo* studies are still required to address many aspects of therapeutically used MSCs, including safety concerns of especially long-term effects, engraftment capability and rejection. It has been speculated that rejection of allogeneic MSCs might be profitable in some instances, because in this way MSCs would only temporarily suppress the immune system, thereby reducing the risk of infection, malignant transformation, or suppression of a graft-versus-tumor effect (Nauta and Fibbe 2007).

Although the mechanisms regarding how MSCs regulate immune cells *in vivo* have not been clearly defined, and controversial reports of *in vitro* and *in vivo* effects exist, their immunosuppressive properties have already been evaluated in investigational clinical settings. The most advanced clinical use of MSCs is to minimize the effects of steroid-resistant graft versus host disease (GvHD) caused by

hematopoietic stem cell transplantation (HSCT). The therapeutic effect on the symptoms of GvHD was first described by Le Blanc and colleagues, simultaneously describing the immunosuppressive effects of MSCs *in vivo* (Le Blanc et al. 2004). Similar results were also obtained by others (Kebriaei et al. 2009). Le Blanc’s group has also published a phase II clinical trial report assessing the influence of MSCs to GvHD of 55 steroid-resistant severe acute GvHD patients, and showed that MSCs can be transferred without HLA-matching (Le Blanc et al. 2008). Also in Finland, GvHD patients have been treated with MSCs. During the year 2013 The Advanced Cell Therapy Center of the Finnish Red Cross Blood Service supplied clinical-grade BM-MSCs products used in the treatment of ten patients suffering from treatment resistant GvHD (Repo 2013 and personal communication). The immunosuppressive effects of MSCs have also been investigated in the treatment of Crohn’s disease, multiple sclerosis, type 2 diabetes mellitus, lung fibrosis, experimental autoimmune encephalomyelitis, and acute pancreatitis (Yi and Song 2012).

### 1.3.3 Mesenchymal stem cells in clinical trials

In February 2014 mesenchymal stem cell transplantation had been studied in 193 clinical trials (<http://clinicaltrials.org>). In 2010 the amount was 105 (Helmy et al. 2010). Helmy and colleagues have reviewed these clinical trials based on their MSC source, whether the MSC transplants were autologous or allogeneic, and listed a variety of diseases MSC transplants have been tested to treat. This data is shown in figure 2.



**Figure 2** Summary of human clinical trials with MSCs. (a) 48 % of cells used were autologous (own) and 42 % allogeneic (donor). (b) MSC transplants have been tested to treat a wide variety of diseases. (c) Most of the MSCs used have been isolated from bone marrow (51%), but cells from the umbilical cord blood (5 %) and adipose tissue (7 %) have also been used. Modified from Helmy et al. 2010.

## **2 GLYCOBIOLOGY**

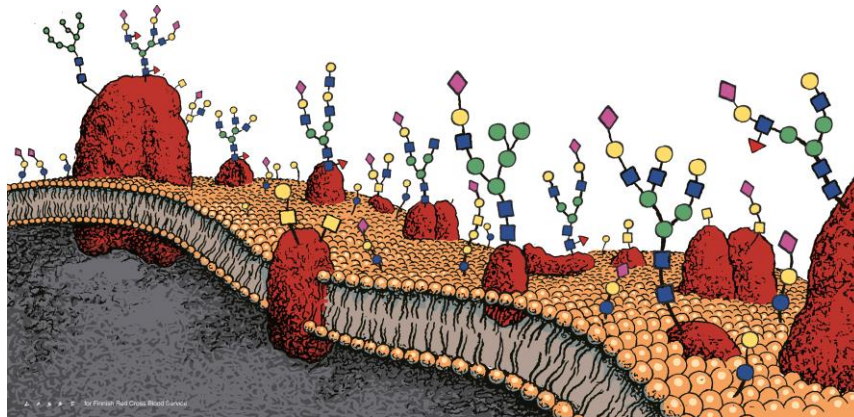
### **2.1 Glycans on the cell surface**

The term glycobiology was created in the late 1980s, and is defined as the study of the structure, biosynthesis, biology, and evolution of glycans that are widely distributed in nature, and the proteins that recognize them (i.e. glycan binding proteins, GBPs) (Varki and Sharon 2009). Before that, in the 1960s and 1970s the studies of other major classes of molecules, such as DNA and proteins, developed rapidly, but the development on methodologies for glycan analysis lagged far behind.

Glycomics is the study of the glycan structures of a given cell type or organism, i.e. the glycome. The glycome is complex and dynamic, changing in development, differentiation, malignancy, or inflammation. Therefore, a given cell type in a given species can manifest a large number of possible glycome states.

#### **2.1.1 Glycocalyx**

The surface of all cells is covered with a dense and complex layer of glycans called the glycocalyx. The diversity of glycan structures is vast and the glycocalyx is characteristic to and different in every cell type. Glycans respond rapidly to intrinsic and extrinsic signals, making the glycome of a given cell dynamic and versatile. Glycans are the first cellular components encountered by approaching cells, pathogens, antibodies, signalling molecules, and other binders. Cell surface glycans have vital roles in many cellular processes, such as adhesion, migration, and signal mediation. They are ideal molecules to be used in isolation, characterization, and identification of different cell populations (Lanctot et al 2007, Cummings 2009). A schematic view of a cell surface is presented in Figure 3.



**Figure 3** Glycan structures (shown with colourful blocks forming linear or forked structures) on the outer side of the cell membrane (orange bilayer) can be attached to proteins (red lumps) forming glycoproteins, or to lipids on the cell membrane forming glycolipids. Image: Lasse Rantanen / Finnish Red Cross Blood Service.

### 2.1.2 Glycan structures

Glycans on the cell surface are extremely variable and complex molecules that are posttranslationally added to proteins or lipids forming glycoconjugates. The type of glycan is defined according to their core structure and the nature of the covalent linkage by which the glycan is attached to its carrier molecule. In glycoproteins, one or more glycans can be attached to polypeptide backbones usually via N- or O-linkages.

All N-glycans share a common core pentasaccharide ( $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}\beta 1$ ) and are attached to the amide group of asparagine residue in the consensus sequence Asn-X-Ser/Thr, where X represents any amino acid except proline. The N-glycan biosynthesis is complicated and highly conserved. N-glycans participate in the folding process and affect many properties of glycoproteins including their conformation, solubility, effector functions, antigenicity, and recognition by GBPs.

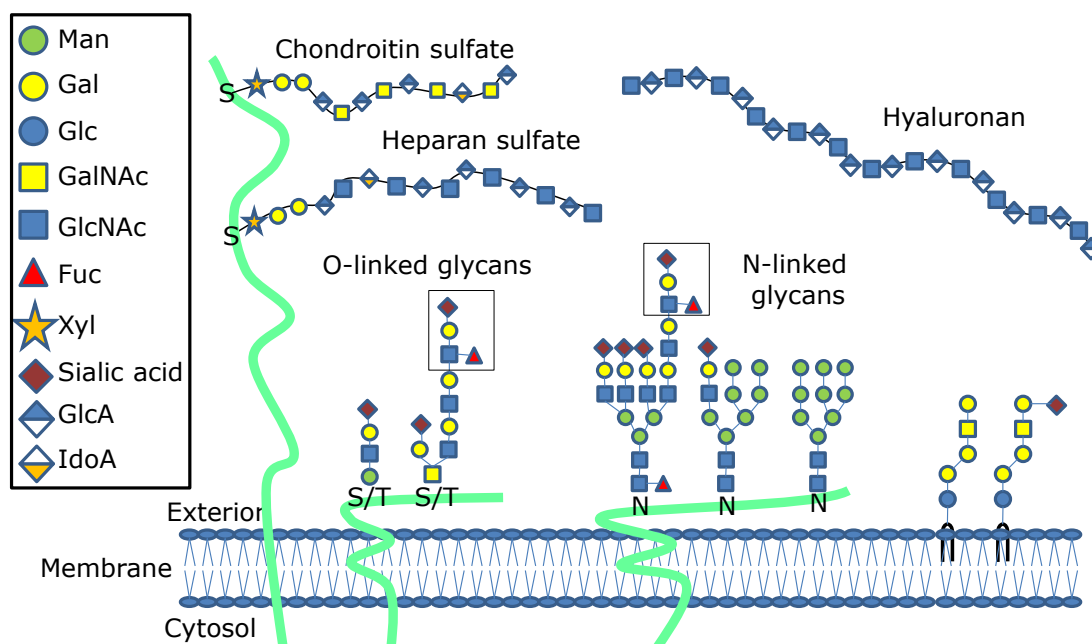
O-glycans are bound to the hydroxyl group of serine or threonine residues of glycoproteins (Brockhausen et al. 2009). There are several types of O-glycans extending into a variety of different structural core classes, including  $\alpha$ -linked N-acetylgalactosamine (O-GalNAc),  $\alpha$ -linked O-fucose (Fuc),  $\beta$ -linked O-xylose (Xyl),  $\alpha$ -linked O-mannose (Man),  $\beta$ -linked O-N-acetylglucosamine (GlcNAc),  $\alpha$ - or  $\beta$ -linked O-galactose (Gal), and  $\alpha$ - or  $\beta$ -linked O-glucose (Glc) glycans. O-GalNAc glycans are called mucin O-glycans and are the most common O-glycan type in mammalian glycoproteins. Hundreds of O-GalNAc glycans with many different extended structures may be attached to mucin glycoproteins. Mucins are ubiquitous

in body fluids and in mucous secretions on cell surfaces where they shield the cell surfaces and protect against infection by pathogens.

Proteoglycans are glycoconjugates in which large sulfated glycosaminoglycan chains with unique core regions are attached to the hydroxyl groups of serine residues. Hyaluronan, a large nonsulfated glycosaminoglycan, does not occur covalently linked to proteoglycans, but can bind to them noncovalently via hyaluronan-binding motifs. Proteoglycans and glycosaminoglycans have many essential functions on the cell surface and in the extracellular matrix (Couchman and Pataki 2012).

In addition to glycans attached to proteins, glycoconjugates can also be composed of glycans attached to lipids. Nearly all glycolipids in vertebrates are glycosphingolipids. They consist of a glycan usually attached to a lipid moiety called ceramide via Glc or Gal. All glycosphingolipids are synthesized from a common precursor, lactosylceramide ( $\text{Gal}\beta 1\text{-4Glc}\beta\text{Cer}$ ) (Chester 1998). Sialic acid containing glycosphingolipids are called gangliosides. Glycosphingolipids are essential in development and they mediate and modulate intercellular coordination in multicellular organisms (Hakomori 2003). Also glycosylphosphatidylinositols (GPI) are a type of glycolipids that covalently attach to proteins and serve as their membrane anchors. Structures of the most common glycoconjugates on the cell surface are shown in figure 4.

Although different glycan classes have unique core regions, by which they are distinguished, the outer structural sequences can be shared among different classes of glycans. These structures often determine the functions or recognition properties of glycoconjugates.



**Figure 4** Common classes of animal glycans. Abbreviations: mannose (Man), galactose (Gal), glucose (Glc), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose (Fuc), xylose (Xyl), glucuronic acid (GlcA), iduronic acid (IdoA). Terminal structure sialyl Lewis x (sLex) both in N- and O-glycans is highlighted with a black box. Modified from Varki and Sharon 2009, Fuster and Esko 2005, Moremen et al. 2012.

Additional diversity in glycoproteins is created by microheterogeneity. This means that all the possible glycosylation sites in the polypeptide are not necessarily glycosylated. Furthermore, a range of variations can be found in the structures of the attached glycans, so that different glycoforms of the same protein exist. The vast diversity existing in glycan structures emerges from the amount of monosaccharide residues, different linkages between them, branched structures, and modifications of monosaccharides, such as phosphorylation, sulfation and O-acetylation.

Glycan structures present in the cell can not be predicted directly from gene expression. Glycosylation takes place in the endoplasmic reticulum and Golgi and each glycan structure is a product of sequential action of competing glycosyltransferases and glycosidases in a subcompartmentalized assembly-line in the ER and the Golgi. Therefore, the composition of glycans is affected by the availability of monosaccharides, and the presence of specific glycosyltransferases and glycosidases in the organism's glycosylation machinery. Defects affecting components of the glycosylation machinery within the cell can cause severe or lethal developmental disorders called congenital disorders of glycosylation (Freeze and Schachter 2009).

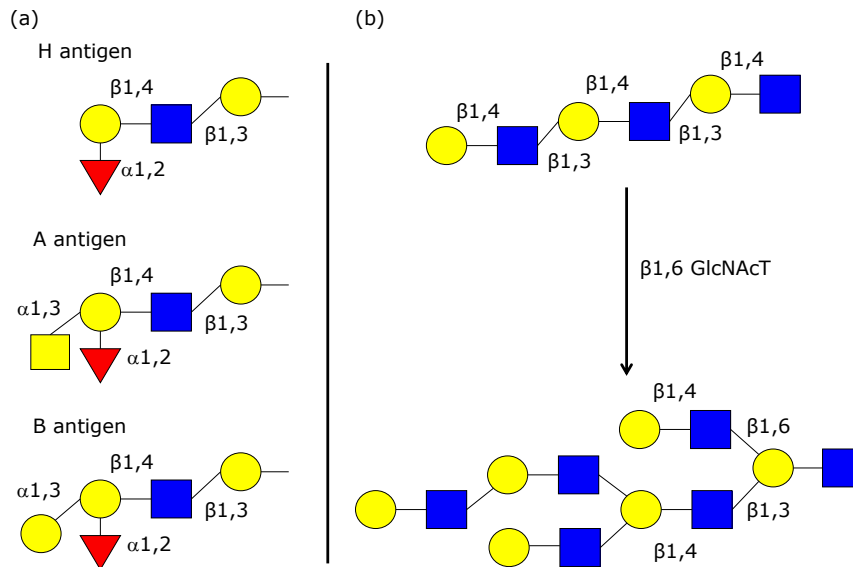
## 2.2 Cellular glycobiology

Glycans on the cell surface are optimally positioned to participate in the communication with the environment. Glycans have many diverse roles in various physiological systems, some of which are briefly described below.

### 2.2.1 Blood group antigens on red cells

At the moment there are 33 known blood group systems on the surface of red cells, seven of which are glycans (Reid et al. 2012). The ABO blood group of an individual is determined by the inherited genes coding different glycosyltransferases resulting in different glycan structures on red cell surfaces. The blood group A glycan epitope is formed by enzyme called  $\alpha$ 1-3GalNAcT encoded by the A allele of the ABO locus. The blood group B allele of the ABO locus encodes the  $\alpha$ 1-3GalT enzyme that forms the blood group B glycan determinant. O alleles at the ABO locus encode a functionally inactive A/B glycosyltransferase and the antigen on the cell surface is called the H antigen. The difference in glycan structures of blood group A and B is only one monosaccharide, yet the clinical relevance of this difference is huge. The endogenous antibodies to specific glycan structures in one person can cause rejection of blood transfusions from another. The terminal structures forming H, A, and B antigens can be part of different glycoconjugates and different core glycan chains in different cells. In figure 5a antigens are shown on type-2 N-acetyllactosamines (LacNAcs), as they are present on red blood cells.

Another carbohydrate blood group antigen system on red cell surface is the i/I antigen. The i antigen, a linear poly-LacNAc chain, is abundantly expressed on the surface of embryonic red blood cells. During the first 18 months of life red blood cells start to express branched poly-LacNAc chain, I antigen, and the level of i antigen declines to very low levels. This developmental regulation is presumed to be due to regulated expression of  $\beta$ 1-6 N-acetylglucosaminyltransferases (I  $\beta$ 1-6GlcNAcT), enzymes responsible for the branching of poly-LacNAc chain. The expression of i/I antigens is not restricted to red blood cells and are found on N-, and O-glycans and on glycolipids (Cooling 2010).



**Figure 5** (a) Type-2 H, A, and B antigens that form O, A, and B blood group determinants, respectively. (b) Type-2 linear poly-LacNAc chain (i antigen) and branched poly-LacNAc (I antigen). Modified from Stanley and Cummings 2009. Blue rectangle, GlcNAc; yellow circle, Gal; yellow rectangle, GalNAc; red triangle, Fuc

### 2.2.2 Selectins in leukocyte rolling

Leukocytes migrate from the circulation to the inflamed tissue as part of the innate immune response. Before extravasation from blood to the tissue, the rapidly moving leukocytes need to slow down. This step is called rolling and is highly dependent on glycan interactions. The endothelial cells in the inflamed tissue express P- and E-selectins. Both of these selectins bind to a specific glycan in a glycoprotein called P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the surface of leukocytes. The glycan structure involved in the binding is sialic acid and fucose containing glycan sialyl Lewis x (sLex) on a specific core 2 O-glycan on PSGL-1. L-selectin, expressed on all leukocytes, is involved in leukocyte homing to secondary lymphoid organs and sites of inflammation. It also binds to sLex glycan, but binding specificity is somewhat different than the binding specificities of P-, and E selectin, e.g. sulfation is required for L-selectin binding. As a characteristic feature of protein-glycan interactions, the sLex-selectin interactions are of low affinity leading to transient attachments of the leukocytes to the vessel wall, i.e. rolling. Through their  $\beta$ 2-integrin (CD11/CD18), slowly rolling leukocytes are able to bind to ICAM-molecules, expressed only in the inflamed tissue endothelial cell. This protein-protein interaction is of high affinity and allows the leukocyte to attach to the vessel wall and invade to the inflamed tissue (McEver et al. 1995).



### **2.2.3 Sialyl Lewis x in fertilization**

The reproductive process is affected by glycans and GBPs. In order to the fertilization to occur, sperm must bind to the translucent matrix covering the oocyte, known as the zona pellucida. After binding, sperm must transit through this matrix to enter the perivitelline space, the space between the zona pellucida and the cell membrane of an oocyte, where they fuse with the oocyte and form a zygote. The interaction between mouse gametes have been shown to be glycan-mediated (Litscher et al. 1995, Wassarman 1990). Although a complete molecular understanding of human gamete binding is not yet available, it is known that the mammalian gamete binding is primarily mediated by the interaction of an egg-binding protein (EBP) on the sperm plasma membrane with carbohydrate sequences on glycoproteins of the egg's zona pellucida (Pang et al. 2011). It has been demonstrated that the sLex is profusely expressed on human zona pellucida glycans and that the binding of sperm can be inhibited with soluble sLex (Pang et al. 2011, Clark 2013). SLex is a well-known selectin ligand, but there are controversial reports of the expression of selectins in the human sperm. It has been suggested that the major egg-binding protein in sperm is very likely a lectin with a binding specificity that overlaps with the selectins (Pang et al. 2011). Substantial evidence has also implicated selectin-mediated adhesions in the early implantation of the embryo (Clark 2013).

### **2.2.4 Cell surface glycans in microbial binding**

In order to infect host cells, microbes often use GBPs to recognize and bind to glycans and glycoconjugates, most commonly sialylated and fucosylated structures on the surface of the host cell (Imberty and Varrot 2008). The binding can be highly selective, demonstrated by sialic acid specific influenza viruses. The influenza virus hemagglutinin binds to sialic acid containing glycans on the cell surface and infects the cell. Human influenza A and B viruses bind to glycans terminating with  $\alpha$ 2-6-linked N-acetylneuraminic acid (Neu5Ac), widely present on the epithelial cells of trachea. Chicken influenza viruses bind to glycans terminating with  $\alpha$ 2-3-linked Neu5Ac, and porcine influenza viruses can bind both types of the aforementioned linkages. In addition, influenza C virus binds exclusively to 9-O-acetylated Neu5Ac (Skehel and Wiley 2000). Rotavirus, the most common cause of severe diarrhea (gastroenteritis) among infants and young children, is another example of sialic acid specific viruses (Yu et al. 2012).

The fucosylated ABH antigens, which constitute the molecular basis for the ABO blood group system, are also expressed in salivary secretions and gastrointestinal epithelia in individuals of positive secretor status. 20 % of caucasians are non-secretors and do not express fucosyltransferase 2, an enzyme needed to convert type-1 LacNAc chains to H antigens in mucus and other secretions (Imberty and Varrot 2008). Many microbes use histo-blood group antigens in the intestinal mucus and other secretions as their binding targets (Wacklin et al. 2011). Norovirus, the common cause of viral gastroenteritis binds to H type-1 antigen and secretor

negative individuals are protected from the infection (Lindesmith et al. 2003). Secretor status is also associated with the composition of some commensal bacteria, such as Bifidobacteria in the human intestine (Wacklin et al. 2011).

### **2.2.5 Differential glycosylation in cancer malignancy**

Glycans regulate many aspects of tumor progression, including proliferation, invasion, angiogenesis, and metastasis. Glycans change in malignant cells as a result of altered glycosyltransferase expression levels and altered location of transferases in the Golgi due to changes in pH (Rivinoja et al. 2009, Hassinen et al. 2011). The changes in glycosylation include both under- and overexpression of naturally-occurring glycans, as well as expression of glycans normally restricted to embryonic tissues (reviewed in Fuster and Esko 2005, Dube and Bertozzi 2005). The common changes include increased  $\beta$ 1-6-branching in N-glycans, overexpression of glycosphingolipids (especially gangliosides), and overexpression of some terminal glycan epitopes commonly found on transformed cells, such as sLex, Globo H, Lewis y (Ley), and Lewis a (Lea). Also mucins are overexpressed in many cancer cells and secreted mucins in the bloodstream can be detected by monoclonal antibodies as an indication of cancer. Another abnormal feature of carcinoma O-glycans is incomplete glycosylation resulting in the expression of Tn, sialylated Tn (sTn), and T antigens. Increased amount of sTn is known to correlate with increased tumour invasiveness and metastatic potential.

In addition, many classes of malignant tumors express high levels of hyaluronan, a nonsulfated glycosaminoglycan that interacts with several cell surface receptors, especially CD44. These interactions are often crucial to tumor malignancy and are current target for novel therapies (Toole 2009, Misra et al. 2011). Also heparan sulfate proteoglycan (HSPG) has been implicated in tumor pathogenesis (Gomes et al. 2013). HSPGs can also bind and store growth factors that can be mobilized by tumor heparanases (Fuster and Esko 2005, Dube and Bertozzi 2005).

The described changes in glycosylation are good markers of cancer and specific GBPs play a crucial role in cancer diagnostics. A few glycan-based targeting strategies have been tested in clinical trials (Fuster and Esko 2005, Dube and Bertozzi 2005, Toole 2009, Misra et al. 2011).

## 2.3 Glycan binding proteins

Glycans are recognized by specific GBPs. Many of the specific biological roles of glycans are mediated via recognition by GBPs. They can be classified to lectins, glycan-specific antibodies, and glycosaminoglycan binding proteins. GBPs are an invaluable tool in the study of glycans, because of their specific binding properties, ease of availability and manageable prices.

### 2.3.1 Lectins

Lectins (lat. *legere*; to pick out or choose / to select) are carbohydrate-specific agglutinins of nonimmune origin. They typically have relatively low affinities for single-site binding and biologically relevant lectin recognition often requires multivalency of both the glycan and GBP, to generate high avidity of binding. Lectins are found in all organisms and they have shared evolutionary origins. Many viral lectins are sialic acid specific and highly selective. Bacterial lectins are either adhesins on the bacterial cell surface that facilitate bacterial adhesion and colonialization, or secreted bacterial toxins (Sharon and Lis 2004).

Plant lectins were found at the end of the 19<sup>th</sup> century and were referred to as hemagglutinins, or phytoagglutinins based on their ability to agglutinate erythrocytes (Sharon and Lis 2004). The initial discovery dates back to 1888 when Stillmark found that the seeds of the castor tree (*Ricinus Communis*) could agglutinate red blood cells (H. Stillmark, 1888). This extract was called ricin and it is both an agglutinin and a very potent toxin. It is now known to bind to cells via interactions with  $\beta$ -linked Gal/GalNAc. In 1940s William Boyd and K.O. Renkonen made independent discoveries that hemagglutinins are ABO blood group specific (Boyd and Reguera 1949, Renkonen 1948). Morgan and Watkins later showed that the binding of these blood group specific lectins could be inhibited by free lectin specific sugars. This finding was among the earliest evidence for the presence of glycans on cell surfaces and indication of the potential roles of glycans as identity markers (Morgan and Watkins 1953).

Lectins have now been found in almost every plant species studied and they are particularly abundant in the seeds of leguminous plants. Natural intrinsic ligands of plant lectins are largely unknown. The ability of plant lectins to recognize many animal glycans with a broad range but high degree of specificity, and their commercial availability, has made them an invaluable tool in the study of human glycans.

Plant lectins were found to recognize glycans on the surface of animal cells in the 1950s, but it took a while before endogenous lectins that recognize these glycans, were identified. The first mammalian lectin, galactose specific hepatic asialoglycoprotein receptor, was isolated 1974 (Ashwell and Morell 1974). Most

animal lectins can be classified on the basis of shared sequence characteristics of their carbohydrate recognition domains (CRDs). Several different structural families are known to exist, including C-type lectins (including selectins), galectins, I-type lectins (including siglecs). Examples of these three lectin types of great importance in cellular interactions are presented below.

Selectins are type-1 membrane proteins having membrane-distal C-type lectin domain. The selectin family contains three members. L-selectin is expressed on mature leukocytes and HSCs, E-selectin on cytokine activated endothelial cells and P-selectin on the surface of activated platelets and endothelial cells. Selectins are crucial in lymphocyte homing, inflammation and immune responses, wound repair, and tumor metastasis. The transient and low-affinity glycan-selectin interactions are important to get the rapidly moving leukocytes to slow, which is a prerequisite for extravasation from blood to the inflammation site. All selectins bind to glycans with terminal  $\alpha$ 2,3-linked sialic acid and  $\alpha$ 1,3-linked fucose, typified by the sialyl Lewis x (sLex) determinant (NeuAc $\alpha$ 2,3Gal $\beta$ 1,3[Fuc $\alpha$ 1,3]GlcNAc $\beta$ 1-R). The main physiological ligand for P-selectin is PSGL-1 containing sLex on a specific core 2 O-glycan. PSGL-1 is expressed on leukocyte surfaces (McEver 2002).

The most widely occurring family of animal lectins is galectins (formerly S-type lectins), so called because they bind to  $\beta$ -galactose containing glycoconjugates with their homologous CRDs of about 130 amino acids. They are found both inside and outside the cell and are multifunctional proteins involved in several cellular functions. Intracellular galectins are involved in mRNA splicing, apoptosis and the regulation of the cell cycle (Liu et al. 2002). Extracellular galectin functions are generally mediated by glycan interactions and include cell-cell, cell-matrix, and protein interactions through glycoprotein and glycolipid binding. These interactions on the cell surface can also mediate signaling inside the cell. Galectins have roles in immune responses and inflammation, development, and tumor metastasis (Rabinovich and Toscano 2009, Camby et al. 2006, Liu and Rabinovich 2005). Galectins lack membrane anchoring domains, but the secreted galectins can be tethered to their ligands in the same or adjacent cells (Stowell et al. 2009).

Even though all galectins bind to  $\beta$ -galactose containing LacNAc structures, their binding preferences are different (Stowell et al. 2008, Horlacher et al. 2010). Galectin-1 preferentially binds terminal LacNAc units of polylactosamines in the branches of multiantennary N-glycans (Camby et al. 2006, Stowell et al. 2008). Galectin-3 can bind both to terminal and internal LacNAc units of a glycan and its affinity has been shown to increase with the amount of LacNAc units, making the i antigen (linear poly-LacNAc) a high-affinity ligand (Stowell et al. 2008). Notably, MSCs express galectin-1 and galectin-3 at high levels, and they have been suggested to be responsible for the immunosuppressive properties of MSCs (Sioud et al. 2011).

Siglecs are sialic acid binding lectins that belong to I-type lectins. I-type lectins are characterized by variable numbers of extracellular immunoglobulin-like domains

and thus belong to the immunoglobulin superfamily, other members of which are antibodies and T cell receptors. There are two subgroups of siglecs; human CD33 related siglecs and conserved siglecs which in humans contain sialoadhesin (siglec-1), CD22 (siglec-2), MAG (myelin associated glycoprotein, siglec-4), and siglec-15. All siglecs are type-1 membrane proteins. Siglecs show restricted pattern of expression in unique or related cell types. With the exception of few members, siglec expression has been found mainly in the hematopoietic and immune systems. For example siglec-2 is expressed only in B cells and regulates multiple B cell functions including cellular activation thresholds and survival. Each siglec has a unique specificity for sialylated ligands, e.g. siglec-2 is unique in having a strong preference for  $\alpha$ 2-6-linked sialic acids. Most siglecs on the cell surface are bound to abundantly expressed sialic acids on the same cell (cis interactions) and are therefore masked. Sialidase treatment, cellular activation or another cell or pathogen bearing higher affinity ligand can unmask the siglecs and result to interactions with other cells (trans interactions). Siglecs are thought to promote cell-cell interactions and regulate the functions of cells in the innate and adaptive immune systems (Crocker et al. 2007).

### **2.3.2 Glycan specific antibodies**

Antibodies belong to the immunoglobulin superfamily and are produced by B cells as part of the immune response. Antibodies are the first line of defense against pathogens and remove dysfunctional or malignant cells. Each individual has circulating antibodies towards a vast repertoire of non-self glycan structures (Huflejt et al. 2009). Amongst the first well-studied anti-glycan antibodies in humans are the anti-blood group antibodies (Watkins 1966).

Antibodies are an essential tool in the research due to their availability, high affinity, and specificity. Specific antibodies have had an indispensable role in the development of protein research. In glycan research, anti-glycan antibodies are an invaluable tool and widely used in glycobiology, but there are more challenges concerning their availability, affinity, and specificity.

#### **2.3.2.1 Antibody isotypes**

There are five different immunoglobulin isotypes, or classes (IgA, IgD, IgE, IgG, and IgM), in mammals. The isotype is dependent on B cell development and activation. The immunological response to carbohydrates is T cell independent, generally resulting in the production of IgM antibodies of low affinity. Even though applicable in *in vitro* research, IgM antibodies are not suited for *in vivo* diagnostics or therapy. Glycan specific antibodies used as research reagents are most often produced in mice, but can be produced in a wide variety of different animals, e.g.

highly specific anti-Neu5Gc antibody of IgY class produced in chicken (Nguyen et al. 2005, Tangvoranuntakul et al. 2003).

### 2.3.2.2 Availability

The availability of anti-glycan antibodies is far less satisfactory than the availability of anti-protein antibodies. This is probably due to the greater challenges in anti-glycan antibody production and the more limited availability of carbohydrate antigens, which are more difficult to purify compared to proteins, and expensive and laborous to synthesize.

### 2.3.2.3 Production

There are several approaches to generate antibodies to glycan antigens. Hybridoma technology is a common method used to generate specific monoclonal antibodies. In this technology, mice are immunized with the carbohydrate antigen after which the mouse splenocytes (containing antibody producing B cells) are fused with myeloma cells to produce hybridoma cells. The hybridoma cells can produce uniform monoclonal antibodies in a cell culture, a great advantage compared to the production of polyclonal antibodies (Köhler and Milstein 1975, Tomita and Tsumoto 2011). Antibodies to carbohydrate structures are more difficult to develop by immunization than anti-protein antibodies because carbohydrates tend to be poor immunogens. For this reason, mice are often immunized with natural glycoconjugates or glycans coupled to carrier proteins to increase their immunogenicity (Huhle et al. 1997, Nozaki et al. 2010), or with whole cells having natural glycans present as a glycoprotein, glycolipid, or proteoglycan on the cell surface (Numata et al. 1990, Xu et al. 2010). Mice can also be infected with parasites or bacteria to generate specific monoclonal antibodies to pathogen-specific glycan antigens (Maruyama et al. 2009). Knockout mice lacking specific glycoconjugates have been used to generate antibodies against the missing glycoconjugate using the antigen in question to immunize the mice (Chen et al. 2000). Hybridoma technology has been especially widely used to generate antibodies against glycan determinants in different types of cancer cells. The immunological response to carbohydrates is T cell independent, thus carbohydrate antigens produced by immunization are often IgM class, which limits their use in therapeutic applications.

An alternative for the generation of human antibodies is antibody phage display technology (Schirrmann et al. 2011). It is completely independent from immunization and thus allows the generation of antibodies against poorly immunogenic molecules or even self-antigens, and uses an *in vitro* selection process. In this technology antibody phage display libraries are constructed by cloning amplified variable heavy ( $V_H$ ), variable light ( $V_L$ ) chains from populations of lymphocytes into phagemid vectors. Different combinations of these domains are displayed on the surfaces of filamentous bacteriophages, each displaying a single

antibody species. Due to limitations of the *E. coli* folding machinery, only antibody fragments like single chain variable fragment (scFv) or antigen binding fragments (Fabs) are used routinely for antibody phage display. The phages binding to a specific antigen are selected from this repertoire. *In vitro* isolation of antibody fragments from antibody gene libraries by their binding activity is called “panning”. Soluble antibodies having the specific binding epitope can be produced in infected bacteria cells and their affinity of binding can even be improved by random mutation, mimicking the affinity maturation in the immune system (Winter et al. 1994). Antibody phage display technology has been successfully used to generate several anti-carbohydrate antibodies, such as antibodies against blood group B (Chang and Siegel 2001), Lewis x (Lex) and sLex (Mao et al. 1999), ganglioside GM3 (Lee et al. 2002), and glycosaminoglycan fragments (van Kuppevelt et al. 1998, Smits et al. 2006). Using this technology, it has been possible to produce completely human monoclonal antibodies from both immune and nonimmune sources, rendering recombinant antibodies a promising source of tools, not only to basic carbohydrate research but also to diagnostic or therapeutic uses.

#### **2.3.2.4 Specificity**

Anti-glycan antibodies can recognize specific glycan structures in the middle of glycan chain or terminal epitopes. The antigen recognized can consist of several monosaccharides, only one monosaccharide, or both glycan and the polypeptide sequence of a glycoprotein or a proteoglycan. Glycan specific antibodies can be highly specific, e.g. antibody to Neu5Gc can distinguish between Neu5Ac and Neu5Gc differing with only one oxygen (Tangvoranuntakul et al. 2003). The specific epitope of antibodies recognizing the same glycan structure can be different, as has been demonstrated with different anti-sLex antibodies (Kannagi and Hakomori 2001).

In order to be used as a tool in research or therapeutic applications, the specificity of a GBP need to be accurately and precisely determined, but detailed epitope characterization of anti-glycan antibodies and lectins can be challenging. Methods to analyze the GBP specificity include X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, isothermal calorimetry, mono- or oligosaccharide inhibition studies, ELISA-type assays and glycan based microarrays.

## **2.4 Use of glycan binding proteins**

### **2.4.1 Glycan binding proteins as reagents in the study of glycans**

Lectins and anti-glycan antibodies are widely used tools in glycobiology. They are mostly used as research reagents in many glycobiological applications such as in the identification of glycans and enrichment and purification of glycoproteins. Lectins are generally cheaper than antibodies, and many lectins currently used as tools in

glycobiology come from plants and are commercially available. Most lectins have broad specificity and are primarily used to monitor general changes of carbohydrate expression. For example Concanavalin A (ConA) binds only to N-glycans and has highest affinity to oligomannose type. However, some lectins have highly accurate binding specificities demonstrated with lectins differentiating between A and B blood group antigens or between  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids. The advantage of antibodies is that unlike lectins, they can be produced against desired glycan structures. Antibodies generally have high specificity for their antigens, but different antibodies to same glycan structures can have different binding epitopes and affinities (Kannagi and Hakomori 2001). This is why the specificity should be accurately determined in order to use the binder to produce high quality information.

The expression levels of glycans are difficult to determine using methods such as gene expression analysis, since glycans are not direct gene products. The presence and amount of glycans in cells or tissues have primarily been monitored indirectly by probing the binding of anti-glycan antibodies and lectins by immunochemistry, flow cytometry and Western blotting (Manimala et al. 2007). Glycolipids can be detected by using GBPs in thin layer chromatography. Flow cytometry using specific GBPs or magnetic beads coupled to GBPs can be used to isolate and sort cells from heterogenous samples. The multivalency of some GBPs can be used to agglutinate cells bearing specific glycan antigens. GBPs are also commonly used in affinity chromatography methods, such as lectin columns, to enrich the glycoprotein before mass spectrometric analysis.

#### **2.4.2 Glycan binding proteins in diagnostics and therapy**

The use of GBPs in diagnostics and therapy is much more limited than their use as research reagents. There is a tremendous potential of GBPs to be valuable diagnostic and therapeutic tools in the future, but it is proven to be very challenging to develop these agents. In order for this to happen, the function mechanism of glycans needs to be elucidated more, and the general glycobiological expertise needs to be improved even more. However, some GBPs are currently used in diagnostics and several antibodies have already been tested in clinical trials of antibody based therapy (Glennie and Johnson 2000). Some of the glycan markers and GBPs used in diagnostics are described below.

One of the best known examples of GBPs in diagnostic is the ability of lectins to recognize blood group antigens on the cell surface (Renkonen 1948). Blood banks, including FRC Blood Service, use specific anti-glycan antibodies to determine groups of red cells. However, some lectins are still used as a back-up strategy in routine blood group screenings.

The other diagnostic use of GBPs with enormous potential is the cancer diagnostics. Glycosylation changes in cancer cells compared to normal healthy cells, and specific glycan markers for cancer have been identified (see section 2.2.5)



(Fuster and Esko 2005). The serological markers CA125, CA19-9, and CA 15-3 are mucin glycoconjugates commonly overexpressed in ovarian, pancreatic, and breast cancers, respectively. Although these and other glycan markers are currently used clinically as sensitive markers for recurrence of disease following initial treatment, they might also be used to facilitate the timing of glycan-based therapies in future cancer treatment programmes (Fuster and Esko 2005).

The other example of clinically relevant glycome change in cancer is the human chorionic gonadotropin (hCG), a glycoprotein hormone normally produced during pregnancy. It is also expressed in certain malignancies, especially by trophoblastic and testicular germ cell tumors, for which hCG is a sensitive marker. The glycosylation of hCG is different in cancer and pregnancy and the glycoform expressed in malignancy is called “hyperglycosylated”. A specific monoclonal antibody B152 used to recognize the hyperglycosylated hCG specifically recognizes a core-2 glycan on Ser-132 and on surrounding peptide structures in malignancy associated variant of hCG. A lectin assay detecting increased fucosylation could also be used in the diagnostic (Valmu et al. 2006).

The carbohydrate deficient transferrin (s-CDT) has been reported to be one of the best laboratory markers in serum for detection of alcohol abuse. Transferrin is a glycoprotein synthesized mainly in the liver. It has two N-glycans containing sialyl residues, tetrasialotransferrin being the most common form. The transferrin glycoforms were originally thought to represent deficiencies in the terminal sialylation (hence the nomenclature). However, these modifications are now known to be more extensive such that entire glycan chains are absent reflecting a more profound effect of alcohol upon liver glycosylation mechanisms (Valmu et al. 2005, Flahault et al. 2003). Immunonephelometric method used to determine the amount of s-CDT is based on the measurement of scattered light which determines the size, shape, and concentration of the scattering particles, in this case the antigen-antibody complexes. Specific antibody recognizing the change in glycosylation is used in the assay (Delanghe et al. 2007).

### **2.4.3 Challenges related to glycan binding proteins**

The quality of the information obtained from antibody and lectin-binding studies depends largely on the specificity of the binders. Even though possessing great potential as research reagents and in diagnostics, improvements are needed in the determination of specificity of GBPs. In addition to accurate specificity, the affinity of most carbohydrate-binding antibodies should be enhanced. When monitoring carbohydrate expression for diagnostic purposes, different studies frequently report conflicting results. As a result, only a small number of carbohydrate antigens are used clinically as biomarkers (Manimala et al. 2007).

It is generally known that glycan antibodies are difficult to generate and may display broad specificity. A carbohydrate microarray profiling of 27 commercially

available antibodies with known specificities demonstrated that many of the antibodies displayed inappropriate binding relative to the listed specificity. More than half of the antibodies studied crossreacted with other glycans on the array (Manimala et al. 2007). The problem with specificity determination is that some anti-glycan antibodies may recognize their antigens only in a specific context, such as on cell surface as part of specific glycoconjugates. A high-throughput microarray analysis has also been performed with 24 lectins and it showed some unexpected binding properties. However, lectins frequently exhibit secondary binding requirements beyond simple mono- or disaccharide specificity, making their specificity hard to analyze (Manimala et al. 2006).

Stage-specific embryonic antigens SSEA-3 and -4 are among the most commonly used markers to identify embryonic stem cells. SSEA-3 (Gal $\beta$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc) is a precursor molecule for SSEA-4 (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc). The most commonly used anti-SSEA-4 antibody (clone MC813-70) has been reported to cross react with GM1b and GD1a glycosphingolipids, with terminal epitope Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (Kannagi et al. 1983). These glycosphingolipids (GSLs) have been shown to be present both in ESCs and in cells differentiated from them (Liang et al. 2010, Liang et al. 2011), suggesting that this anti-SSEA antibody may not be the best one to detect undifferentiated embryonic stem cells. This highlights the importance of knowing what other antigens the antibody binds in addition to the one it is generated against. Strategies combining different methods, such as GBPs in flow cytometry or immunostaining combined with mass spectrometric analysis, can be used to verify results obtained with only one method (Liang et al. 2010, Liang et al. 2011).

It has also been reported that there is considerable heterogeneity in the carbohydrate specificity of anti-sLex antibodies, which adds complexity to selectin-mediated adhesion analyses. sLex determinants can be part of different glycan chains, have differentially bound sialic acids or have additional sulfate modifications. Different antibodies recognize these slightly different epitopes differently. Some anti-sLex antibodies also cross react with closely related sLea structure (Kannagi and Hakomori 2001).

Antibody specificity variations in glycans binding can have significant implications for biomarker performance as demonstrated comparing five different sLea antibodies used in pancreatic cancer detection. Glycan array analysis revealed that certain antibodies were highly specific for the sialyl Lewis a (CA19-9) epitope, while others bound also a related but non-fucosylated structure called sialyl Lewis c. The use of antibody with broader specificity led to the detection of an increased number of pancreatic cancer patients without increasing the detection of pancreatitis (Partyka et al. 2012). This highlights the value of both characterizing the accurate specificity of antibodies and other binders, as well as detecting the accurate antigens elevated in cancer or other disease conditions.

## **3 GLYCANS IN STEM CELL BIOLOGY**

### **3.1 The glycan markers of stem cells**

Glycans in the glycocalyx comprise a prominent molecule class on the cell surface, which is why many commonly used stem cell surface markers are glycoproteins, glycolipids, and proteoglycans. Enzymes involved in the glycan synthesis can also be used as markers.

#### **3.1.1 Glycome profile**

The glycome profile of a cell is characteristic to a certain stem cell type and can be used to identify different kinds of stem cells. Mass spectrometry has been used in the analysis of the glycome profile of different types of stem cells. A study comparing the N-glycans of undifferentiated human ESCs and their differentiated progeny demonstrated that the N-glycome on the cell surface reflects cell's differentiation stage (Satomaa et al. 2009). It was seen that the most characteristic glycosylation feature in the undifferentiated human ESCs was complex fucosylated structures, i.e. fucoses in the antennae of N-glycans, such as Lex and H type 2 antennae in sialylated complex-type N-glycans. The N-glycan structures of HSCs have also been analyzed with mass spectrometric profiling (Hemmoranta et al. 2007). Human hematopoietic stem cells (CD133+) were shown to have enriched amount of biantennary complex-type N-glycans, high-mannose-type N-glycans and increased terminal  $\alpha$ 2-3-sialylation level compared to progenitor cells (CD133-). Information from the glycan profiles is useful when identifying stem cells from differentiated progenitor cells and also when developing future strategies regarding stem cell targeting.

#### **3.1.2 Surface antigens**

Individual glycan structures are commonly used markers when identifying specific cell types. GBPs are commonly used tools to analyze specific cell surface glycan determinants, commonly in flow cytometric analysis or immunofluorescent staining. GBPs can also be used in a wide variety of different methods, such as affinity chromatography and ELISA-based methodology, or in enrichment of cell populations known to have certain glycan antigens on their surface. Panels of surface markers can be used to monitor differentiation status of stem cells. Human ESCs can be characterized with a panel of pluripotency associated cell surface markers, including glycan antigens SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (International Stem Cell Initiative et al. 2007). Also MSCs are characterized by a panel of markers suggested by ISCT (Dominici et al. 2006). These markers are almost all glycoproteins. Whether these glycoproteins need to be correctly glycosylated in order for MSC to have specific functions remains still unknown. The heavily

glycosylated sialomucin molecule CD34 is commonly used marker for human hematopoietic stem cells. CD133 is another cell-surface glycoprotein used to identify human HSCs, although it seems to identify a slightly different and more primitive subset of HSCs than CD34 (Hemmoranta et al. 2006). CD133 is also used to identify and purify multipotent neural stem cells (Uchida et al. 2000).

Human ESCs have specific glycosphingolipid (GSL) profiles (Liang et al. 2010, Liang et al. 2011). Patterns of GSL expression change greatly during development and differentiation. In mass spectrometric analysis, human ESCs have been shown to have globo- and lacto-series GSLs on their surface, but the cells switch to ganglio-series GSLs when they differentiate. The switch was shown to be the result of altered expression of glycosyltransferases in the biosynthetic pathways of the GSLs (Liang et al. 2010). Lactoseries GSLs found on human ESCs include fucosyl Lc4Cer (Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcCer), bearing H type 1 antigen (underlined in the structure), which is the precursor of A, B, and Lewis blood group antigens. H-type 1 epitope is present in undifferentiated ESCs and disappears during differentiation (Liang et al. 2010). Commonly used ESC markers SSEA-3 and SSEA-4 are epitopes on the globo-series GSLs, termed GL-5 and GL-7 (Kannagi et al. 1983). SSEA-3 and SSEA-4 are found to be present in ESCs and their amount decreases rapidly upon differentiation (Liang et al. 2010). Even though the amount of SSEA-3 and SSEA-4 on the ESC surface diminishes when the cell differentiates, it has been shown that they are not essential for the maintenance of human ESC pluripotency (Brimble et al. 2007).

Sialic acids are typically found at the outermost end of glycan antennae and sialylated structures on various macromolecules are recognized by many cell-type-specific antibodies. Polysialylated neural cell adhesion molecule (PSA-NCAM) is a glycoprotein and a prominent cell-surface glycan marker. PSA is a quite unique carbohydrate structure composing of linear homopolymer of  $\alpha$ 2-8-linked-N-acetylneuraminic acid and its presence on NCAM is developmentally regulated. PSA-NCAM is involved in many aspects of neurogenesis and plasticity (Bonfanti 2006).

The tumor-rejection antigens (Tra) are widely used markers of ESCs. The monoclonal antibodies Tra-1-60 and Tra-1-81 are known to recognize carbohydrate epitopes and are routinely used to assess the pluripotency status of human ESCs and iPS cells (International Stem Cell Initiative et al. 2007). It has been suggested that both antibodies recognize keratan sulfate proteoglycan, but the binding of Tra-1-60 is dependent on sialic acids, and binding of Tra-1-81 is not (Badcock et al.1999). However, the glycan array analysis suggested specific binding of Tra-1-60 and Tra-1-81 to terminal type-1 lactosamine epitopes present in human ESCs as part of a mucin-type O-glycan structure (Natunen et al. 2011).

### 3.1.3 Glycosyltransferases

In addition to cell surface antigens, glycosyltransferases involved in the glycan synthesis can also be used as stem cell markers. The mRNA expression of glycosyltransferase genes can be measured and used as an indication of the differentiation state of the cell. The N-glycan biosynthesis pathway is composed of sequential addition (or removal) of monosaccharides in the form of nucleotide sugars, each catalyzed by a specific glycosyltransferase (or glycosidase). The expression level of specific glycosyltransferases defines the glycan structures present on the cell surface.

In a study comparing the N-glycan structures and associated gene expression in human hematopoietic stem and progenitor cells, many stem cell specific transferases and corresponding glycan structures were determined (Hemmoranta et al. 2007). MGAT2 gene, encoding GlcNAcT2 glycosyltransferase, was shown to be overexpressed in undifferentiated HSCs. This enzyme catalyzed the addition of GlcNAc residue to the second antenna of the forming N-glycan, an essential step in the conversion from oligomannose- to complex-type N-glycans. As a result of this enzyme activity, biantennary complex-type N-glycans were enriched in HSCs. Also, the MGAT4 gene, encoding GlcNAcT4 was shown to be underexpressed in HSCs compared to progenitor cells. GlcNAcT4 catalyzes the addition of GlcNAc residue to a mannose residue in the forming N-glycan, resulting in the formation of triantennary N-glycans. HSCs were also shown to have elevated  $\alpha$ 2-3 sialylation, supported by the overexpression of ST3GAL6 gene encoding  $\alpha$ 2-3 sialyltransferase. Another sialyltransferase,  $\alpha$ 2-6 sialyltransferase competes for the same substrates with  $\alpha$ 2-3 sialyltransferase and the sialylation type in the N-glycan surface is a result of the expression level of specific sialyltransferases.

It was seen from the N-glycan profile of the human ESCs that the most characteristic glycosylation feature in the undifferentiated human ESCs was complex fucosylated structures. Fucosyltransferase genes FUT1, -2, -4 and -8 were shown to be expressed in human ESCs. When compared to differentiated embryoid body cells FUT1 and FUT4 were overexpressed. Lex and H type-2 structures formed by the action of glycosyltransferases encoded by FUT4 and FUT1, respectively, were clearly recognized from the profile (Satomaa et al. 2009).

SSEA-4, a widely used marker of ESCs has also been seen in the surface of MSCs derived from cord blood (Suila et al. 2011). The gene expression analysis showed that the expression of ST3Gal-II, which is the SSEA-4 synthase, was clearly elevated, correlating well with the amount of SSEA-4 on the cell surface.

In addition to determining of the expression of individual glycosyltransferase enzymes and the corresponding glycan structures, the development of more thorough transcript profiling methods has been started. The transcript profiling of glycan-related genes has its own set of complexities and mapping enzymes to complex glycan biosynthetic pathways for glycoprotein, glycolipid and proteoglycan

biosynthesis and catabolism are still in their early stages. The fact that many of the critical enzymes involved in the glycan modifications are encoded by a relatively low amount of transcripts, brings additional complexity to the study of glycan-related gene expression. However, some analytical platforms have been developed (Nairn et al. 2010, Nairn et al. 2012).

## 3.2 Role of glycans in stem cell cultures

Cell culture is a crucial step that can have dramatic effect on cells, especially to the antigens on the cell surface. Bone marrow transplantations are normally done without any cultivation of the HSCs, but in the development of advanced cellular therapy products, such as MSCs, the cells are isolated, expanded and maybe even manipulated before used in the future therapeutic approaches. Therapeutic cells should optimally be cultivated in xeno-free conditions, meaning animal derived materials should not be used. Changes in the glycans on the cell surface are a good indication of the use of animal derived materials during the culture.

### 3.2.1 Lectin as embryonal stem cell culture matrix

Human embryonic stem cells used to be, and still often are, cultured using fetal mouse fibroblasts as feeder cells. The use of mouse fibroblast feeder cells inhibits the spontaneous differentiation of human ESCs *in vitro* and removal of the feeder cells leads to enhanced differentiation (Reubinoff et al. 2000). Optimal feeder-free culture matrices for human ESCs have been under development and include Matrigel<sup>TM</sup>, or laminin-coated plates and conditioned mouse feeder-cell medium (Xu et al. 2001). These approaches both have some challenges since they contain animal derived materials compromising potential clinical applications. ESCs have also successfully been cultured on xeno-free systems containing human-derived feeder cells. Human fetal and adult fibroblast feeders from human fetal muscle, fetal skin, and adult fallopian tubal epithelial cells have been shown to support prolonged undifferentiated human ESCs (Richards et al. 2002).

Since glycans on the cell surface have important roles interacting with the cell's environment, lectins binding to the glycan epitopes commonly found from the human ESC surface could be considered as an optimal culture matrix (Mikkola et al. 2013). Indeed, *Erythrina cristacalli* agglutinin (ECA), specific for type 2 LacNAc structures, was shown to support the self-renewal capacity of the cells in long-term culture. The expression of pluripotency markers and functional characteristics of the cells cultured on ECA were comparable to cells on Matrigel<sup>TM</sup>. ECA introduces as a fully defined, promising and, cost-effective cell culture matrix for human ESCs.

### 3.2.2 Non-human glycans in cultured stem cells

The culture media of many cells typically contain fetal calf or bovine serum (FCS/FBS), or in some cases horse serum. Human ESCs and MSCs are commonly cultured in the presence of animal-derived materials for research purposes. When stem cells are cultured for therapeutic applications, animal derived medium components need to be replaced. However, the clinical efficacy of MSCs in human diseases has been investigated using MSCs cultured with FCS in a number of clinical trials (Horwitz et al. 2002, Bang et al. 2005). The animal derived components in the culture medium of cells have been shown to alter the glycosylation. Human ESCs grown in contact with mouse feeder cells and animal-derived cell culture medium components have Neu5Gc on their surface (Martin et al. 2005). The two most common mammalian sialic acids are Neu5Gc and Neu5Ac, with Neu5Ac being the metabolic precursor of Neu5Gc. As a consequence of inactivating mutation in CMP-N-acetylneuraminic acid hydroxylase (CMAH) enzyme, Neu5Gc is not present in humans (Varki 2001). Neu5Ac differs structurally from Neu5Gc with only a single oxygen atom. Human cells can take Neu5Gc from cell culture and cell separation reagents such as FCS, serum replacement, and bovine serum albumin (BSA) (Martin et al. 2005). In addition to stem cell cultures, human cells take up Neu5Gc from their surroundings containing animal products of even from dietary sources such as red meat and milk products, and metabolically incorporate it to the forming glycans using the same Golgi transporter and sialyltransferases as CMP-Neu5Ac (Tangvoranuntakul et al. 2003, Bardor et al. 2005). The human immune system recognizes Neu5Gc as a foreign and produces circulating anti-Neu5Gc antibodies (Tangvoranuntakul et al. 2003, Martin et al. 2005). Thus, using stem cells cultivated in the presence of animal derived material could compromise transplantation success by inducing an immune response. It has been reported that the Neu5Gc xenoantigen contamination in human ESCs as well as in MSCs is reversible, and Neu5Gc can not be detected from the cell surface after the cells are transferred to animal-free culture conditions (Heiskanen et al. 2007). The Neu5Gc content of cultured human cell lines and their secreted glycoproteins can also be reduced by adding excess of Neu5Ac to the culture medium (Ghaderi et al. 2010).

Other non-human glycan detected in human ESCs is the  $\alpha$ -galactosyl epitope ( $\alpha$ -Gal). Human and other primate cells do not express the  $\alpha$ 1-3 galactosyltransferase capable of making the linkage (Macher and Galili 2008).  $\alpha$ -Gal is highly immunogenic and humans have natural preformed antibodies against the epitope.  $\alpha$ -galactosyl epitope is present on mouse fibroblast feeder cells used in ESC cultures. The epitope is not metabolically incorporated into human cells but carried along into hESC preparations (unpublished results).

Because of the immunogenic nature of non-human glycan structures, they introduce remarkable risk in stem cell transplantation.

### 3.2.3 Metabolic glycoengineering

As cell surface glycosylation is affected by the culture conditions, the properties of therapeutic cells can be manipulated through metabolic glycoengineering. In this technique monosaccharide analogs are added to culture medium through which they are introduced into the metabolic pathways of a cell and are biosynthetically incorporated into the glycocalyx. However, it has been shown that adding unnatural sialic acid precursor can influence not only the sialic acid composition of cell surface glycans, but also the overall N-glycan profile of the cells (Natunen et al. 2013). These changes may affect the properties of MSCs and need to be considered when the glycosylation is manipulated, but when carefully controlled, could also be utilized for example in the optimization of stem cell biodistribution.

## 3.3 Glycosylation in stem cell biodistribution

Glycosylated structures on the cell surface have vital roles in many cellular processes such as adhesion, migration, proliferation, and signal mediation (reviewed in Lanctot et al. 2007, Cummings 2009). A major issue in the use of various types of stem cells for regenerative therapy is to achieve sufficient localization of the cells to the desired sites of tissue damage.

Homing is the process by which cells migrate to, and engraft in, the tissue in which they can exert their local functions. Cell migration involves a cascade of events initiated by adhesive interactions between flowing cells and the vascular endothelium at the target tissue. These interactions result in cell-tethering and rolling contacts on the endothelial surface, typically followed by chemokine-triggered activation of integrin adhesiveness, firm adhesion, and extravasation (Sackstein 2005).

### 3.3.1 Glycans in hematopoietic stem cell homing

Transplanted HSCs engraft the bone marrow and migrate to the sites of damage, such as stroke and myocardial infarction, by means of the concerted action of specific surface molecules that mediate homing from vascular to tissue compartments. Similarly to leukocytes, the migration of HSCs is selectin dependent (Hidalgo et al. 2002). Selectin ligands expressed in HSCs are PSGL-1 and HCELL (hematopoietic cell E-/L-selectin ligand), a specific glycoform of CD44 glycoprotein. PSGL-1 is expressed on various mature leukocytes, on several hematopoietic cell lines, and on primitive human hematopoietic stem and progenitor cells. HCELL expression is characteristic only for primitive hematopoietic cells (Dimitroff et al. 2001). Both PSGL-1 and HCELL have terminal sLex (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuca1-3)GlcNAc) structures, mediating the interaction with selectins (Foxall et al. 1992). In PSGL-1 the sLex epitopes are in O-glycans and in HCELL in N-glycans (Sackstein 2004). It has been noticed that impaired fucosylation of the cell



surface glycans results in the lack of rolling and homing of the cells (Xia et al. 2004, Hidalgo and Frenette 2005).

### 3.3.2 Glycans in mesenchymal stem cell homing

MSCs have been shown to home to site of the injury or inflammation and to produce paracrine factors with anti-inflammatory properties, resulting in functional recovery of the damaged tissue (Salem and Thiemermann 2010). The mechanisms of MSC migration is not as well known as the mechanisms of leukocyte or HSC homing. MSCs are a heterogenous population of cells, which causes controversy in the results of studies involving “homing receptors” of MSCs. It has been shown that only a small portion of MSCs in one transfusion experiment had the capacity to home to bone marrow (Wynn et al. 2004). Different sources of MSCs can also have differences in the expression of surface antigens causing variability to homing procedure and creating additional controversy to the results (Nystedt et al. 2013). It is also good to keep in mind that the surface structures of the cells are dynamic and differences in culture conditions, such as plating density, confluence, passage number, and cytokine supplements cause alterations and make it more challenging to compare different cell products.

MSCs have been shown to express many protein surface antigens involved in the homing cascade, including integrins, chemokine receptors, and CD44 (Henschler et al. 2008, Qu et al. 2014). However, unlike with leukocytes and HSCs, the role of selectins in MSC homing remains elusive. MSCs have been shown to display coordinated rolling and adhesion behavior in a P-selectin dependent manner. The P-selectin ligand in MSCs is still undefined, since these cells have shown not to have the expected ligand, PSGL-1, bearing sLex on a specific core 2 O-glycan, on their surface (Rüster et al. 2006). A recent study suggests a novel interaction between two lectins, P-selectin and galectin-1 on UCB-MSCs (Suila et al. 2014). Whether this interaction is dependent on carbohydrates and the lectin properties of these two proteins needs further elucidation.

MSCs have also been shown to lack the expression of E-selectin ligands (Sackstein et al. 2008). MSCs do have CD44 on their surface, but a different glycoform than HCELL in HSCs. The glycans of the CD44 in MSCs have terminal  $\alpha$ 2-3 sialic acids linked to galactoses, but they lack the  $\alpha$ 1-3-linked fucose modifications on GlcNAc residues needed to form sLex (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-R), a vital part of the epitope binding to E-selectin. Treating MSCs with fucosyltransferase VI (FTIV) to create HCELL has been shown to result in E-selectin-mediated homing of intravenously injected human MSCs to bone (Sackstein et al. 2008) and prime the cells for transendothelial migration, which is needed for entry into the site of inflammation or injury in any intravenous application (Thankamony and Sackstein 2011). The rolling response of human MSCs both on P-selectin coated substrates *in vitro* and on inflamed endothelium *in vivo* has also been achieved by chemically incorporating sLex to the cell surface

(Sarkar et al. 2011). MSCs derived from the umbilical cord blood (UCB-MSCs) have been reported to have higher fucosylation level and more sLex on their surface than MSCs derived from bone marrow (BM-MSCs) (Nystedt et al. 2013). This and other differences in the adhesion molecules on these two MSC types were shown to influence the biodistribution of the cells.

In addition to carrying glycan ligands essential to selectin-mediated homing of MSCs, CD44 can also function as a lectin that binds hyaluronan, a large nonsulfated glycosaminoglycan (Lesley et al. 2000, Qu et al. 2014). The CD44-hyaluronan interaction has been shown to mediate the migration of MSCs into wound sites (Zhu et al. 2006, Bian et al. 2013).

# SUMMARY OF THE STUDY

## 4 AIMS OF THE STUDY

Glycosylation on the cell surface is a characteristic and cell-type specific feature of all cells, and GBPs are a valuable tool in the characterization of the cell-surface glycans. Novel stem cell markers and well characterized GBPs binding them are needed in the research of stem cell glycosylation as well as in therapy applications.

The purpose of this thesis was to characterize and develop glycan binding proteins keeping in view of their use as tools in MSC therapy. The specific aims were:

- ❖ characterization of glycans on the surface of mesenchymal stem cells
- ❖ scrutinizing different GBPs, including 8 lectins and 69 antibodies, in the characterization of MSCs
- ❖ characterization of linear poly-LacNAc, i.e. the i antigen, as a UCB-MSC marker
- ❖ production of i blood group antigen (i.e. linear poly-LacNAc) -specific antibody with recombinant technology
- ❖ production of MSC surface glycan specific antibodies with whole cell immunization and hybridoma technology

## 5 MATERIALS AND METHODS

### 5.1 Methods

The materials and methods used in this study are described in detail in the original publications and listed in Table 1. The original publications are referred to using their Roman numerals I-IV.

**Table 1.** Materials and methods used in the study

<b>Method</b>	<b>Used in study</b>
MSC culture	I, II, III, IV
- preparation of MSC lines from BM or UCB	I, II, III, IV
- MSC differentiation	I, II, III, IV
- enzymatic treatment of cell surface proteins and glycans	I, II, III, IV
- chemical derivatization of cell surface glycans	IV
Isolation of red blood cells	III
Flow cytometry	I, II, III, IV
Immunofluorescence staining and microscopy	I, II, III
mRNA expression analysis	II
Amino acid sequence analysis and comparison	III
Mass spectrometry	I, II
- glycan and glycoprotein isolation for mass spectrometry	I, II
- mass spectrometric analysis of lipid linked-, N- and O-glycans of the cell	I
- mass spectrometric analysis of cell surface N-glycans	II
mass spectrometric data analysis	I, II
Phage display antibody production	III
- construction of scFv phage display libraries	III
- selection and screening of recombinant antibodies	III
- preparation of phage clones	III
- red blood cell agglutination assay	III

Oligosaccharide competition assay	II, III
Hybridoma technology for antibody production	IV
- immunization of mice	IIV
- hybridoma cell preparation and culture	IV
- monocloning of hybridomas	IV
- Dissociation-enhanced lanthanide fluorescent immunoassay (DELFI A)	IV

## 5.2 Ethics

This study was approved by the ethical review board of the Finnish Red Cross Blood Service and Helsinki University Hospital (statement numbers 550/E8/02 and 235/13/03/00/2011). Umbilical cord blood was obtained after normal vaginal delivery with full consent of informed and healthy volunteers at the Helsinki University Central Hospital, Department of Obstetrics and Gynecology, and Helsinki Maternity Hospital. Only UCB units unsuitable for therapeutic use were used in these studies. The most common reason for discharging UCB units for therapeutic use was a small total volume or a low total number of cells.

## 6 RESULTS

The main results of the studies are summarised here. The results are presented and discussed in detail in the accompanying original publications, which are referred to using their Roman numerals I-IV.

### 6.1 Characterization of mesenchymal stem cell glycome (I, II)

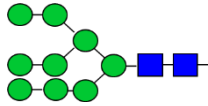
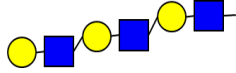
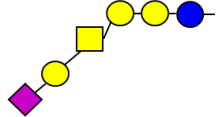
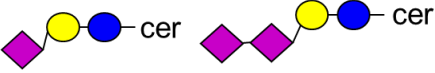
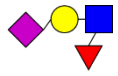

Stem cell glycosylation has been shown to have unique features. However, the majority of the studies have concentrated on embryonic stem cells (Muramatsu and Muramatsu 2004). We wanted to widen the research on human stem cell glycosylation into MSCs. To get a comprehensive conception of the cellular glycome of MSCs, we combined mass spectrometric profiling and GBP analysis to study MSCs both from bone marrow (I) and from umbilical cord blood (II).

First the glycome profiles of both BM-MSCs and corresponding osteogenically differentiated cells were analyzed. Comparison of the profiles revealed that the stem cells and osteoblasts both have characteristic glycosylation features and that the glycome changes during osteogenic differentiation occur in a predetermined fashion. The mass spectrometric analysis showed that high-mannose N-glycan structures and fucosylated acidic O-glycans were more dominant in stem cells than in differentiated osteoblasts. All major glycosphingolipid classes were found in both MSCs and differentiated osteoblasts, but MSCs expressed more small ganglio-type glycans than osteoblasts. Further analysis revealed that MSC N-glycans contain fucosylated terminal structures, such as Lex. Also sialylation, mainly  $\alpha$ 2-3-linked sialic acid, was shown to be a typical structure on MSCs. It was also shown that MSCs have linear poly-LacNAcs in N- and O-glycans and possibly also in glycosphingolipids. This structure was not seen in differentiated osteoblasts. Differentiated osteoblasts contained more complex- and hybrid-type N-glycans, sulfate modifications, and larger glycosphingolipids than undifferentiated MSCs.

Since the mass spectrometric profiling of MSC glycome encompassed the overall cellular glycome, including intracellular glycans, further verification of cell surface presentation of the observed glycan structures was obtained by cell surface cytochemical staining and flow cytometric analysis with GBPs, either lectins or antibodies. In agreement with the mass spectrometric data, MSCs were shown to contain more mannosylated structures than their osteogenic counterparts, shown with *Hippeastrum hybrid* lectin (HHA) staining. The *Solanum tuberosum* lectin (STA) recognizing mainly linear-poly-LacNAc chains was shown to pronouncedly stain MSCs (I, Figure 5c,d), verifying the mass spectrometric result. GBP analysis also clearly showed that MSCs have increased amount of sialyl Lewis x on their surface, compared to differentiated cells (I, Figure 5a,b and figure 6 c,d). This is in good

agreement with the findings of the mass spectrometric analyses showing fucosylated and  $\alpha$ 2-3-sialylated structures in both N- and O-glycans. In flow cytometric analysis, MSCs, but not differentiated cells, were also shown to have SSEA-4 on their surface (I, Figure 6a,b). SSEA-4 is a widely used embryonic stem cell marker, also observed in MSCs (Suila et al. 2011). However, SSEA-4 was not observed in mass spectrometric analysis. The analysis of glycome profiles and the verification of glycan structures are more thoroughly presented in publication I. Table 2 shows examples of the glycans enriched in undifferentiated mesenchymal stem cells.

**Table 2.** The glycans enriched in undifferentiated mesenchymal stem cells. Only one high-mannose type structure is shown in the table, even though structures with different number of mannose residues were seen in the profile. Green circle, Man; blue rectangle, GlcNAc; yellow circle, Gal; yellow rectangle, GalNAc; purple diamond, Neu5Ac; blue circle, Glc; red triangle, Fuc.

Glycan enriched in MSCs	Glycan structure
High-mannose type	
Linear poly-N-acetyllactosamines	
SSEA-4	
Shorter ganglioside structures (e.g. GM3, GD3)	
Sialyl Lewis x	
$\alpha$ 2-3-linked sialic acids	

We also performed a mass spectrometric analysis of MSCs derived from umbilical cord blood and made verifications with GBPs (unpublished data, except for linear-poly-LacNAc shown next, II).

## 6.2 The i antigen on the surface of mesenchymal stem cells (I, II)

One of the most interesting findings in the glycan profile of BM-MSCs was that linear poly-LacNAc structures, also called the i antigen, are present on undifferentiated BM-MSCs but not on osteoblasts differentiated from them (I, Figure 3 and Figure 5 c,d). We next showed that the i antigen is also present on the surface of UCB-MSCs. The N-glycans on the surface of UCB-MSCs were analyzed by mass spectrometry and found to include linear di-LacNAc epitope (II, Figure 4).

The expression of i antigen was more thoroughly characterized with GBPs. The lectin binding properties of UCB-MSCs were studied with different LacNAc binding lectins. The *Solanum tuberosum* agglutinin (STA), *Lycopersicon esculentum* agglutinin (LEA) and *Datura stramonium* agglutinin (DSA) lectins mainly recognize linear poly-LacNAc structures. In flow cytometric analysis they all strongly stained UCB-MSCs (98-100 %), indicating the presence of i antigen on the cell surface in great amounts (II Figure 1). STA lectin recognized epitopes also on BM-MSCs in cytochemical staining, whereas the osteogenically differentiated cells were hardly recognized (I fig 5). UCB-MSCs were also shown to have some amount of branched poly-LacNAc structures (the I antigens) on their surface, indicated by the staining with *Phytolacca Americana* agglutinin (PWA) recognizing mainly branched poly-LacNAc structures (II figure 1). Also, 80% of the UCB-MSCs stained positive for terminal LacNAc structures with *Erythrina cristacalli* agglutinin (ECA) (II, figure 1). Chitotriose (GlcNAc<sub>3</sub>) inhibited the binding of STA, DSA, LEA and PWA, verifying the specificity of lectin stainings (II, data not shown).

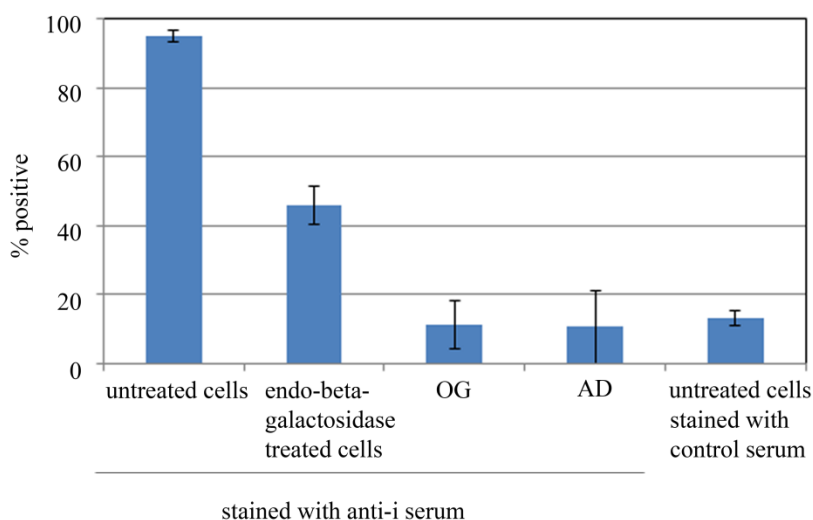
Galectins are a family of animal lectins that bind to LacNAc structures. Galectin-3 can bind both to terminal and internal LacNAc units of a glycan and its affinity increases with the amount of LacNAc units, making the i antigen (poly-LacNAc) a high-affinity ligand (Stowell et al. 2008). We studied the expression of galectin-3 in UCB-MSCs and observed notable mRNA expression of galectin-3. The relative gene expression level of galectin-3 was found to be significantly higher than the relative gene expression levels of the studied MSC markers (II, figure 6a). Galectin-3 on UCB-MSC surface was also detected by mass spectrometry and flow cytometry (II, figure 6b,c).

To obtain further evidence of the presence of i antigen on UCB-MSC surface, we stained the cells with polyclonal human serum containing anti-i IgM immunoglobulins. Flow cytometric analysis revealed that 42-98% of the UCB-MSCs stained positive with anti-i serum in repeated experiments (II, figure 2 a, b). We also discovered that even though anti-i serum strongly stains UCB-MSCs, both the osteogenic and adipogenic cells differentiated from them were almost unstained (II, figure 3). This is in good agreement with our findings of the MS analyses showing the enrichment of i antigen on BM-MSCs, but not in osteogenic cells differentiated



from them (I). The fact that i antigen is present only in undifferentiated mesenchymal stem cells could indicate its potential role as a marker of stemness.

To confirm that the anti-i serum recognizes linear poly-LacNAc structures, UCB-MSCs were treated with endo- $\beta$ -galactosidase, an enzyme that hydrolyses internal  $\beta$ -galactosidic linkages of linear poly-LacNAc structures. The enzyme treatment drastically diminished the amount of cells stained with the anti-i serum (from 98% to 48%), indicating the disappearance of linear poly-LacNAc structures (II, figure 2c). Results of the presence of linear-poly LacNAc on UCB-MSC surface are combined on figure 6.



**Figure 6** Summary of the analysis of linear-poly LacNAc antigen (i blood group) on UCB-MSC surface. Polyclonal anti-i serum stains most of the cells in flow cytometric analysis. The staining is drastically diminished when cells are treated with endo-beta-galactosidase that cleaves linear poly-LacNAc chains. Cells differentiated to osteogenic (OG) or adipogenic (AD) progenitor cells are not stained more than the cells stained with control serum not recognizing the i antigen. The data is a compilation of independent flow cytometric experiments and the error bars represent differences within each experiment.

Furthermore, we showed that several specific glycosyltransferases involved in the synthesis of linear poly-LacNAc structures are expressed in UCB-MSCs (II, figure 5).

## 6.3 Production of glycan binding proteins (III, IV)

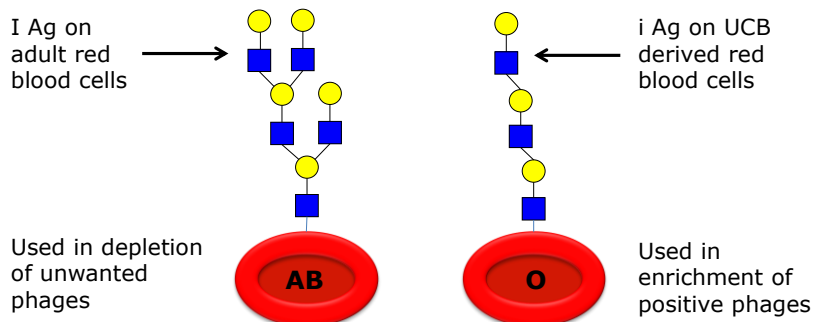
Few anti-i antibodies have been produced in the 80s and 90s (Feizi et al. 1980, Fenderson et al. 1986, Hirohashi et al. 1986, Miyake et al. 1989, Nagatsuka et al. 1995), but none of them are commercially available anymore. None of the antibodies produced are not available anymore. Since the production of an anti-glycan antibody is not as easy as the production of anti-protein antibodies, we decided to approach it with two different techniques (summarized in table 3). The first approach was to

## SUMMARY OF THE STUDY

make a specific anti-i recombinant antibody with phage display technology (III), and the second approach was to make a mesenchymal stem cell surface specific monoclonal anti-glycan antibody with whole cell immunization technology (IV).

The challenge in anti-i antibody production was to find a blood donor with an elevated serum anti-i titer. Detection of a suitable donor made it possible to construct IgM phage display libraries from donor lymphocytes (III, figure 1). Since synthetic glycans do not necessarily exactly resemble the naturally occurring glycan chains in the cell surface, we used red blood cells having the natural antigens on their surface to select the potential anti-i binders from the libraries. Umbilical cord blood -derived red blood cells, which naturally have the i antigen on their surface were used in the selection, and adult-derived red blood cells, naturally having the I antigen on their surface, were used in the depletion of the libraries (figure 7).

By using naturally occurring glycan chains, we wanted to avert the possibility that the antibodies selected using synthetic glycan would not recognize the intended antigen in the form that is present on cell surface. After four affinity selection rounds, about 100 scFv phages enriched to i-positive red blood cells and further characterization provided one that recognized the i antigen on UCB-MSCs (III).



**Figure 7** Red blood cells used in the selection of scFv clones from antibody phage display libraries.

Our another objective was to produce and characterize monoclonal antibodies against UCB-MSC surface glycans with hybridoma technology. We used intact UCB-MSCs to immunize mice and selected glycan-specific antibodies produced by hybridoma cells. Using whole cells in the immunization, it is possible to produce antibodies against previously unknown carbohydrate antigens, specific for the cell type in question.

From the 250 hybridoma culture medium samples containing polyclonal antibodies, five were able to bind to UCB-MSC structures in DELFIA binding assay, three of which were shown to be glycan specific by enzymatic and chemical treatment of glycans. During the monocloning, antibody production of one of the polyclonal hybridoma cultures ceased and we ended up with two monoclonal MSC surface glycan specific antibodies (IV, table 1).

## 6.4 Epitope determination of glycan binding proteins (II, III, IV)

The specificity of a binder is the most crucial character that has to be accurately and precisely determined. However, detailed epitope characterization on GBPs is challenging.

### 6.4.1 Enzymatic and chemical cell surface modification (II, II, IV)

As mentioned above (6.2), the specificity of the anti-i serum was demonstrated by treating the cells with endo- $\beta$ -galactosidase, an enzyme that hydrolyses internal  $\beta$ -galactosidic linkages of linear poly-LacNAc structures (II). The dramatic decrease of the antiserum binding level demonstrated that the serum recognizes linear poly-LacNAc structures, i.e. the i antigens (figure 6 and II figure 2c). Enzymatic treatment of cell surface molecules was also used when examining the capability of prospective anti-i scFv antibodies to agglutinate the i antigen containing UCB-derived red blood cells (III). To enhance the agglutination efficiency and improve carbohydrate accessibility, red blood cells were enzymatically treated with sialidase and papain. Sialidase was selected to remove terminal sialic acids and make repeated lactosamine structures more accessible. However, this treatment was observed unnecessary for the induction of agglutination. In contrast, treatment with papain, a cysteine protease catalyzing the breakdown of proteins, showed that removal of bulk cell surface proteins was critical for the binding of scFv antibodies to red blood cells (III, table 3). Agglutination assays comparing the ability of prospective anti-i scFv antibodies to agglutinate i antigen containing UCB-derived red blood cells but not adult red blood cells that don't have the i antigen on their surface, were essential in the determination of i antigen specific antibodies (III).

When producing MSC surface glycan specific antibodies, we enzymatically removed the cell surface glycans in order to study which antibodies lose the binding to their cognate antigens after removal of the glycans (IV). In order to comprehensively remove different cell surface glycan structures, we used a cocktail of wide variety of enzymes. PNGase F is an endoglycosidase and was used to release N-linked oligosaccharides. O-glycosidase was used to release unsubstituted Ser- and Thr-linked  $\beta$ Gal-(1-3) $\alpha$ GalNAc (core 1 type O-glycan) from glycoproteins. Broad specificity neuraminidase capable of hydrolyzing different types of sialic acid linkages was used to remove the sialic acids, and  $\beta$ 1-4 galactosidase to release  $\beta$ 1-4-linked galactose from the non-reducing end of complex oligosaccharides.  $\beta$ -N-acetylglucosaminidase was used to liberate terminal  $\beta$ -linked N-acetylglucosamine and  $\alpha$ 1-(3,-4) fucosidase to release antennal  $\alpha$ 1,3-fucose and  $\alpha$ 1,4-fucose from complex carbohydrates. Removal of cell surface glycans clearly affected the binding of three of the tested antibodies, but the variations between replicates were quite large. Therefore, we subsequently chemically modified the cell surface glycans with periodate oxidation. Mild periodate oxidation at acidic pH leads to conversion of vicinal hydroxyl groups of glycan chains to reactive aldehyde groups without

altering the structure of polypeptide chains. As the treatment opens the carbohydrate ring, it alters the binding determinants enough to prevent the binding of carbohydrate-specific antibodies. We used 5mM periodate to modify terminal glycans on the MSC surface enabling us to identify three carbohydrate binding and two non-carbohydrate binding antibodies in a DELFIA assay (IV, figure 1). The three carbohydrate binding antibodies were the same as those identified with enzymatic treatment.

All carbohydrate antigenic determinants are not sensitive to periodate treatment. Determinants consisting of linear carbohydrate chains with linkages at carbon 3, such as the i antigen, are considered not to be periodate sensitive (Woodward et al. 1985, Niemann et al. 1978). This explains why we did not see any effect of the periodate treatment of UCB-MSCs on the binding of prospective anti-i antibodies (III).

#### **6.4.2 Competition binding assay (III)**

To find out if the epitope recognized by the prospective anti-i antibodies is a linear polygalactosamine, we performed a competition binding assay with three different galactosamine oligosaccharides (III). A flow cytometric analysis showed that the linear galactosamine containing two LacNAc units and a galactose unit inhibited the cellular binding of all three prospective anti-i antibodies tested (III, figure 5).

#### **6.4.3 Glycan array (III, IV)**

Array formats containing vast variety of different glycans have been developed to test the specificity of GBPs. We tried to screen the carbohydrate specificity of the developed recombinant antibodies, but were not very successful with this method. The prospective anti-i antibodies as scFv hyperphages were sent to the Consortium for Functional Glycomics (CFG) glycan array containing 611 different glycan structures. We did not get any specificities to our binders, but this might be a methodological issue, since the array is optimized for antibodies, not hyperphages (III). Also the two antibodies produced with hybridoma technology were sent to the CFG glycan array, but the very low relative fluorescence signal level of the results did not relate to significant binding to any of the glycans. However, if the antibodies were to recognize both glycan and a peptide epitope, or a glycosaminoglycan epitope, this would not be seen in the CFG glycan array (IV).

#### **6.4.4 Sequence analysis and comparison (III)**

The serum antibodies that bind the i antigen belong to cold agglutinins (Pascual et al. 1992). An immunoglobulin V<sub>H</sub>4.21 gene segment has been shown to be required to encode anti-i and anti-I specificities (Pascual et al. 1992, Schutte et al. 1993, Smith et al. 1995). The similarity of all five prospective anti-i antibodies was between 64 %

and 83 % compared to V<sub>H</sub>4.21 gene segment (III, figure 2). The antibody having highly similar amino acid sequence (83%) was shown to be the most promising anti-i antibody also in other specificity experiments (III).

#### **6.4.5 The occurrence of the epitope (I, II, III, IV)**

Antigens on cell surface can be characteristic to a certain cell type or to a certain stage of the cell development. We showed that glycan antigens on BM-MSCs, the glycan profile, is characteristic to this cell type and changes when the cell differentiates (I).

As the i antigen is known to be present in red blood cells derived from umbilical cord blood, but not in adult red blood cells, this feature was used to identify and isolate anti-i antibodies from all the antibodies in the phage display library (II). The i antigen was also shown to be present on MSC surface, but not on the surface of osteogenic or adipogenic cells differentiated from MSCs (II, figure 3). This revealed that the i antigen can be used as a MSC marker (II). When producing the anti-i antibody, we utilized this quality to select the i antigen binding antibodies (III, figure 4).

Many glycans are known to be species specific. Our monoclonal antibodies were produced using UCB-MSCs in the immunization. The binding to other cell types was observed and the two antibodies also recognized human BM-MSCs, but did not recognize porcine BM-MSCs. Neither did they recognize human HT-29 cancer cells or human fibroblasts, one of the end products of mesenchymal differentiation (IV, table 2).

One indirect way to get information of the glycan antigen recognized by an antibody is to study if the antigen epitope is in a glycoprotein, glycolipid, or proteoglycan. The protease sensitivity of our two monoclonal antibodies produced with hybridoma technology was studied by detaching the UCB-MSCs from culture plates with pronase or trypsin with varying duration and temperature of treatment. Flow cytometric analysis showed that the binding of both antibodies was highly sensitive to treatment with both proteases. Pronase treatment abolished the binding totally and trypsin treatment significantly, depending on the circumstances (IV, figure 2). This indicates that these antibodies might be epitopes in glycoproteins or in proteoglycans.

SUMMARY OF THE STUDY

**Table 3.** Summary of the production of anti-glycan antibodies.

<b>Produced antibody</b>	<b>Production method</b>	<b>Antibody type</b>	<b>Antigen</b>	<b>Antigen specificity determination</b>
Anti-i Ab (III)	Phage display technology (i/I panning)	Recombinant Ab (hyperphage)	linear poly-LacNAc (i antigen)	<ul style="list-style-type: none"> <li>• red blood cell agglutination assay</li> <li>• DNA sequencing</li> <li>• Oligosaccharide competition binding assay</li> </ul>
Anti- MSC Ab1 (IV)	Hybridoma technology (MSC immunization)	Monoclonal Ab (IgG <sub>1</sub> κ)	MSC surface glycoprotein or proteoglycan	<ul style="list-style-type: none"> <li>• Glycosidase treatment of cell surface glycans (mixture of different glycosidases)</li> <li>• Chemical derivatization of cell surface glycans (periodate)</li> <li>• Protease treatment (pronase)</li> </ul>
Anti- MSC Ab2 (IV)				

## DISCUSSION

Glycobiology is a fast developing field of interest studying glycans, molecules vitally involved in every aspect of life. Glycans were once considered merely as decorative elements serving simply structural and energy requirements in a cell. Perhaps the most important reason why the progress in glycobiological research was not as rapid as the progress and enthusiasm in the study of other macromolecules lies in the structural complexity of glycans. However, over the last few decades, new technologies have been developed which have been proven very powerful in glycobiological research (reviewed in Cummings 2009, Varki and Sharon 2009).

Antibodies have played a significant role in the field of protein research and they are widely used in the identification and purification of proteins. GBPs are used in glycobiological research in multiple different methods, such as ELISA, glycan arrays, cytochemical staining, and flow cytometry. The use of GBPs is usually cost-efficient and method development and validation are relatively easy compared to e.g. NMR and mass spectrometry. However, there are some challenges with the use of GBPs (Kannagi and Hakomori 2001, Manimala et al. 2006, Manimala et al. 2007, Liang et al. 2010, Liang et al. 2011). Glycans are poor immunogens often resulting to glycan specific antibodies with weak affinity. Furthermore, the specificity of GBPs is challenging to determine accurately and precisely. The specificities of commercially available GBPs are not always accurate and should not be blindly trusted (Manimala et al. 2006, Manimala 2007, Partyka et al. 2012). At the moment, the results obtained with GBPs should always be verified with other methods to avoid misinterpretation of the data (Liang et al. 2011). The contradictory results between different methods in the same study or between different research groups are likely due to either cell line specific expression of the epitope, differences in the cell isolation or in culture conditions, or differences in the handling of the cells required for different methods. Different binding characteristics of GBPs in solid-phase methods, such as glycan arrays and ELISA, compared to binding of ligands on cells or in solution, also cause differing results.

### **Glycans are potential biological biomarkers to be used in stem cell characterization and therapy**

Glycans play important roles in a vast array of biological processes, such as fertilization, bacterial and viral infections, inflammation, and cancer metastasis (Lanctot et al. 2007). The vast diversity of glycan structures changes rapidly and continuously, responding to intrinsic and extrinsic signals (Cummings 2009). Glycans are at the center of many disorders and diseases, making them important research object both for therapeutic and diagnostic purposes. Realizing the potential and promise that glycobiology holds, many pharma and biotech companies are

novadays allocating their research and development resources to it, glycan engineered therapeutic antibodies as one example.

Another therapeutically interesting field is stem cell research. Stem cells hold enormous therapeutic potential in various medical applications. MSCs are currently in a focus of intense clinical and scientific investigation. They are a promising cell type for various applications in the field of tissue engineering as well as an attractive candidate for therapy of several immune-mediated disorders (Kirkpatrick et al. 2014, English et al. 2010, Bernardo et al. 2012). Unlike HSCs, MSCs are cultured before transplantation to a patient. This makes them biological drugs (called advanced therapy medicinal product, ATMP), controlled by regulatory authorities and requiring marketing authorization. The European Medicines Agency (EMA, [www.ema.eu](http://www.ema.eu)) evaluates and supervises medicinal products in Europe, and in Finland this is regulated by the Finnish Medicines Agency (Fimea, [www.fimea.fi](http://www.fimea.fi)).

The glycans on the cell surface are ideal molecules for identification, purification, and characterization of cells for therapeutic purposes (Lanctot et al. 2007). The effects of culture conditions are seen rapidly on the glycosylation and glycans can even be manipulated to change e.g. the biodistribution of the cell (Nystedt et al. 2013). For these purposes, methods are needed to reliably and proficiently determine the cell surface glycans. GBPs in general serve as diagnostic tools in medical and scientific laboratories. High affinity and exquisite specificity are important factors for their successful use.

In this thesis, our first aim was to analyze the glycome of MSCs and to find novel MSC specific markers. The research was then expanded into developing GBPs for both stem cell and glycobiological research, keeping in mind the therapeutic applications for both fields.

### **Stem cell surface glycans are characteristic to a cell type**

We first analyzed the glycome of MSCs and compared it to the glycome profile of osteogenically differentiated cells (I). Combination of techniques to complement and verify the results were used. It has been a well-tried practice to combine especially the data from mass spectrometry and GBPs to get more reliable results (Liang et al. 2010, Liang et al. 2011). Our data clearly indicates that MSCs have a specific glycosylation pattern that changes when the cell differentiates, thus the glycome profile analysis can be used to evaluate MSC differentiation state. Corresponding results have been obtained from the analysis of ESC (Satomaa et al. 2009) and HSC (Hemmoranta et al. 2007) N-glycomes with mass spectrometry. The glycome analysis also revealed other interesting findings, including the increased expression of linear poly-LacNAc in MSCs compared to osteoblasts differentiated from them. This finding was observed by mass spectrometric fragmentation analysis, digestion with endo- $\beta$ -galactosidase (an enzyme that specifically cleaves linear poly-LacNAc), and staining with GBP, a lectin called STA. Poly-LacNAcs are known to



be expressed in red blood cells in a developmentally regulated manner (Cooling 2010). Linear poly-LacNAc chains (i antigen) in fetal blood cells are changed to branched chains (I antigen) in the adult. Poly-LacNAc structures are ligands for galectins and thus involved in cell adhesion, microbe-host interactions, and modulation of immune responses (Rabinovich and Toscano 2009).

### **Linear poly-N-acetyllactosamine (i antigen) is a marker for mesenchymal stem cells**

The expression of linear poly-LacNAc was further studied in UCB-MSCs and based on the findings, we suggested it as novel marker for these cells (II). Again, a combination of methods was used to confirm the results. Multiple different GBPs were used to recognize the linear poly-LacNAc structure on the cell surface. Lectins specific for linear poly-LacNAc structures, STA, LEA, and DSA, all recognized the structure. Also a polyclonal patient serum containing anti-i IgM recognized the specific structure on flow cytometric analysis. The same serum is used in blood group serology. This antiserum failed to recognize the cells after treatment with endo- $\beta$ -galactosidase, indicating that it specifically recognizes the linear poly-LacNAc structure. Mass spectrometric and mRNA expression analysis supported the results obtained with GBPs. Often the best and most reliable result is obtained when the mass spectrometric glycan profile data is combined with data from GBP studies and verified with the gene expression analysis of glycosyltransferase expression. Linear poly-LacNAc (the i antigen) is developmentally regulated in red blood cells, but the expression of the i and I antigens in other tissues has been noticed not to correlate with the red blood cell phenotype (Thomas 1974, Cooling 2010). However, this study shows that the expression of linear poly-LacNAc structures in MSCs is similar to red blood cells and is regulated according to cell differentiation. Undifferentiated MSCs have linear poly-LacNAc structures on their surface, but these structures are not expressed on the surface of cells differentiated along adipogenic or osteogenic lineages. Therefore, linear poly-LacNAc can be used as a novel MSC marker.

There are currently no commercially available antibodies recognizing the linear poly-LacNAc antigen. In blood group serology, the typing of i antigen on red blood cells is typically performed using polyclonal human antisera, expressing the antibody with high enough titer. None of the anti-i antibodies, listed in Glyco Epitope database ([www.glyco.is.ritsumei.ac.jp/epitope](http://www.glyco.is.ritsumei.ac.jp/epitope)), are available anymore (personal communication with the corresponding writers of Feizi et al. 1980, Fenderson et al. 1986, Hirohashi et al. 1986, Miyake et al. 1989, Nagatsuka et al. 1995). Since an anti-i antibody could be useful both in the identification of MSCs aimed at therapy and in blood group serology, we decided to develop our own anti-i antibody (III). Antibody phage display technology to generate recombinant antibodies was used, to avoid problems related to poor immunogenicity of glycans. This technology has been successfully used to generate several anti-glycan antibodies, including antibody against blood group B (Chang and Siegel 2001). Similar to the study of Chang and

colleagues (Chang and Siegel 2001), we used red blood cells in the panning, to find the specific binders for the antigen in question. In the production and characterization of the binders, they were used in multiple different methods, including agglutination assay, flow cytometric analysis, and competition binding assay. Characterization of potential binders resulted in the selection of one prominent antibody specific for linear poly-LacNAc structure.

### **Mesenchymal stem cell surface glycans introduce other alternative markers**

We also used an alternative strategy to generate novel MSC surface glycan specific antibodies (IV). Glycans are known to be poor immunogens, but the immunogenicity increases when glycans are presented as glycoconjugates, i.e. glycoproteins, glycolipids, or proteoglycans (Fuster and Esko 2005). In this study, we used intact UCB-MSCs to immunize mice and selected glycan specific antibodies produced by hybridoma cells. The glycan specificity was analyzed using periodate oxidation, where glycans on the antigens are oxidized preventing the glycan specific antibody to bind its cognate antigen (Woodward et al. 1985). Using whole cells in the immunization process, it is possible to produce antibodies against previously unknown, natural antigens, specific for the cell type in question. Typically antibodies produced by immunization are IgM class, and therefore not well applicable for *in vivo* diagnostics or therapy (Ravn et al. 2004). However, IgG antibodies from immunization are also known to occur. Both of the two antibodies produced in this study were IgG class. The produced GBPs were used in different methods, such as DELFIA microplate method, flow cytometry, and glycan array, to confirm the results.

In the studies (III and IV) presented in this thesis, we were successful in generating carbohydrate specific antibodies. These antibodies could be used in stem cell research as well as in therapeutic applications. However, the characterization of these GBPs has had many challenges, as has been noticed in many previous studies (Kannagi and Hakomori 2001, Manimala et al. 2006, Manimala et al. 2007, Liang et al. 2010, Liang et al. 2011, Partyka et al. 2012). There are different methods with different advantages for characterization of protein-glycan interactions. ELISA-type solid-phase methods using GBPs are easy and cost-effective to develop, but are laborious and require large amounts of both glycans and GBPs. This problem might be alleviated with the progress of methods to synthesize glycans. Other methods with same shortcomings are NMR spectroscopy and X-ray crystallography which also require special equipment and trained personnel (Manimala et al. 2007). Glycan microarrays are a relatively new technology for high-throughput screening of glycan-GBP interactions. Glycan arrays contain a huge variety of glycans and require much less GBPs than other, more traditional methods. A publicly available glycan microarray has been introduced by the Consortium for Functional Glycomics ([www.functionalglycomics.org](http://www.functionalglycomics.org)). In our studies, we sent both the recombinant antibodies (III) and monoclonal antibodies produced by immunization (IV) to be

analyzed with this glycan array. Unfortunately, we did not get any results for either set of antibodies. Our recombinant antibodies sent were in the form of hyperphages and it is currently unclear if this glycan array is a suitable method for assaying glycan-binding specificity of hyperphages since there is no previous experience in successfully using this technology for hyperphages. Also, the monoclonal glycan-specific antibodies produced by immunization did not bind significantly to any of the glycan structures on the array. This gave us reason to believe that our antibodies bind either to glycosaminoglycan structures missing from the array or both to the glycan and the underlying peptide sequence of a glycoprotein or a proteoglycan. The disadvantage of glycan arrays is that they vary in ligand presentation, the glycans are not necessarily presented in the form as they occur naturally (isolated glycans vs. chemically synthesized), and assay conditions and detection methods can affect the binding or the observed result. Also the immobilization of the glycans on the flat surfaces can modify the recognition capability of GBPs. All these features contribute to the affinity and selectivity of binding, and thus the assay may not reflect the actual conditions and binding on the cell surface (Manimala et al. 2007, Leppänen and Cummings 2010).

## **Future prospects in stem cell glycomics**

Applications using MSCs in therapy are gaining incremental interest. Their capability to suppress immune mediated diseases like GvHD has already been proven in practice (Le Blanc et al. 2004, Kebriaei et al. 2009, Le Blanc et al. 2008). However, the naturally ununiform population of MSCs, different tissues of origin and variations in culture protocols between different research institutions, lead to slightly different populations of MSCs, even though the cells would fulfill the minimal criteria for MSCs (Dominici et al. 2006).

There has been progress in gaining better understanding of the role of glycans in biological processes, earlier hindered by technical limitations in the glycobiological field. Glycans on the cell surface are prominent structures and ideal markers for cells. Methods to analyze glycans need to become widely accessible also to researchers who are not specialists in carbohydrate chemistry, mass spectrometry or NMR. Use of GBPs is easily accessible in many methods, such as cytochemical staining, flow cytometry, or affinity chromatography. The challenges to overcome with GBPs are the poor availability, broad specificity, and low affinity. GBPs are not readily available for numerous glycan structures that exist in nature. The production and characterization of exact specificity are still challenging and the affinity between glycans and proteins is naturally weak. It may take some years until good enough tools to routinely analyze glycan structures on the stem cell surfaces are in wide use in diagnostic and therapeutic applications. But it is promising that the utmost importance of cell surface glycans has been started to be understood in all aspects of cellular biology, not just among glycobiologists.

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