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Human embryonic stem cell N-glycan features relevant to pluripotency

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Abstract: Human embryonic stem cells (hESCs) and reprogrammed human induced pluripotent stem cells (hiPSCs) proliferate indefinitely in the undifferentiated state (self-renewal) and differentiate into the three germ layers (and ultimately into all cell types) (pluripotency). Branching in the N-glycan core of hESCs/hiPSCs is limited. hESCs/hiPSCs have high mannose-type and biantennary complex-type core structures, indicating immature stages of N-glycoproteins. The two branches on the core consist of type 2 N-acetyllactosamine (LacNAc), completed mainly with α 2,6-linked sialic acid. Complex terminal fucosylation, particularly α 1,2-linked fucosylation, is another characteristic N-glycosylation feature of hESCs/hiPSCs. All of these structural features are probably associated with the pluripotency of hESCs/hiPSCs. In differentiated cells, two-, three-, and four-branched N-glycan core structures are seen and N-acetylhexosamine compositions increase in the branches. The elongation and size of the branches in the N-glycan cores increase by the addition of poly-LacNAc chains and are the typical features during differentiation. As α 1,2-linked terminal fucosylation disappears, α 1,6-linked core fucosylation increases. It is necessary to know the pluripotency-associated glycans in order to identify and isolate the pluripotent cells from heterogeneous populations containing differentiated cells.

Key words: Human embryonic stem cell N-glycans, N-glycan core branching, pluripotency, differentiation

1. Introduction

The glycan parts of glycoconjugates and oligosaccharide chains are intricate information-carrying or information-storing biopolymers (André et al., 2015; Hu et al., 2015). They encode extremely rich information in the sugar code, conveyed via noncovalent hydrogen bonds (Cocinero and Çarçabal, 2015; Maverakis et al., 2015). Our knowledge about sugar codes or sugar-based molecular information systems is much less than our knowledge about DNA-protein-based information systems (Gabius et al., 2002; Miron and Petitjean, 2015). Free or membrane-associated glycan binding proteins (GBPs) (lectins, galectins, selectins, siglecs, mannose-binding proteins and receptors, antibodies, and glycosylation enzymes) recognize and interact with certain oligosaccharide motifs of glycoconjugates (Cummings and Pierce, 2014). In this way, GBPs play critical roles in deciphering stored information in sugar codes and translating it into biological responses (Gabius, 2000; Bernardi and Cheshev, 2008; Kaltner and Gabius, 2012; Murphy et al., 2013). The exquisite binding specificities of GBPs determine the composition, sequence, and linkage of glycan structures (Smith and Cummings, 2013). Immunohistochemical labeling results indicate that GBPs are mainly distributed in the cytoplasmic membrane, the perinuclear region of the cytoplasm, and the Golgi area (Zhong et al., 2015). Through this intracellular distribution, GBPs regulate a number of vital processes in which glycan recognition and adhesion interactions occur. For instance, N-glycoprotein processing-related events such as protein folding (Bieberich, 2014; Kaszuba et al., 2015), protein sorting (Liang et al., 2014), and protein degradation (Quan et al., 2008; Słomińska-Wojewódzka and Sandvig, 2015) are performed in these regions using the recognition interactions of GBPs (lectins) through related glycan codes or signals. The sugar code recognition specificity of GBPs can also potentially help determine the stem cell glycophenotype.

Stem cells are undifferentiated cells with high capacity for self-renewal and pluripotency. Their self-renewal property allows embryonic stem cells to proliferate indefinitely in their undifferentiated state, whereas their pluripotency indicates their differentiation capacity into the three germ layers, the endoderm, mesoderm, and ectoderm, and into all cell types of the adult body (Kraushaar et al., 2013). Depending on their origin and differentiation potential, two types of stem cells are distinguished in humans: human embryonic stem cells (hESCs) and human somatic or induced pluripotent stem cells (hiPSCs)

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(Nakamura, 2008). hiPSCs are reprogrammed somatic cells for the pluripotent state via the introduction of certain transcription factors through cell reprogramming technology (Takahashi et al., 2007; Alisson-Silva et al., 2014). hESCs and hiPSCs are attractive sources for cell-replacement therapies. Unfortunately, the tumorigenic propensity (Itskovitz-Eldor, 2011) of stem cells is a major obstacle in clinical applications. A new surface marker, stage-specific embryonic antigen-5 (SSEA-5), that is highly and specifically expressed on H type-1 glycans of hiPSCs (Tang et al., 2011) was recently determined. An antibody, namely anti-SSEA-5, recognizes this highly specific surface marker on undifferentiated hiPSCs in cell culture; when applied to incompletely differentiated hESC cultures prior to cell transplantation, the risk of teratoma formation is removed through the immunodepletion of SSEA-5 and two additional markers (Tang et al., 2011). It is clear that specific markers are required for the discrimination (Rosu-Myles et al., 2013), isolation (Oliveira and Barreto-Filho, 2015), enrichment (Nakatsu and Deng, 2013), and elimination of tumor initiating cells (Tang et al., 2011) for the effective utilization of stem cells. Glycans are useful tools for this purpose (Lanctot et al., 2007; Yanagisawa, 2011). Cell surface glycans are part of glycoconjugates (glycoprotein, proteoglycans, glycosphingolipids, and glycophosphatidylinositol anchors) and are the most appropriate markers to discriminate stem cells from nonstem cells; moreover, they are also probably associated with self-renewal (Nakamura, 2008; Higashi et al., 2014; Pulsipher et al., 2015).

Several changes on the surface glycosylation pattern occur during differentiation of ESCs (Draper et al., 2002; Wearne et al., 2006; Satomaa et al., 2009; Dodla et al., 2011; Wang et al., 2011). The altered glycosylation pattern disrupts previous cellular interactions and initiates new interactions between stem cells and their microenvironments. Characteristic surface glycosylation patterns of differentiated cells, glycotypes, provide a molecular signature and serve to discriminate the differentiated cells from each other even during the differentiation stages (Varki et al., 2011; Dodla et al., 2012; Nairn et al., 2012). Some glycan modifications affect the fate of ESCs. Specific glycans cause ESCs to undergo an accelerated exit from self-renewal and differentiate into neuronal cell types (Pulsipher et al., 2015).

There are several methods available to determine the altering glycophenotype of specific glycan structures in stem cells during differentiation (Pulsipher et al., 2015). Previous studies on glycosylation pattern characterization of stem cell surfaces were conducted with labeled lectin applications on glycosidase digested or nondigested cells, using microscopic methods (Venable et al., 2005; Wearne et al., 2006, 2008). Useful impressions of mainly terminal and some internal monosaccharide residues of oligosaccharide chains were obtained from these studies. Advances in analytical technology, including nuclear magnetic resonance (NMR) and mass spectrometry, have provided additional structural information about stem cell glycosylation (Satomaa et al., 2009). Newly developed microarray technology has also been used to analyze the glycans of stem cells (Tateno et al., 2011; Toyoda et al., 2011).

The current review focused on the cell surface N-glycan features of hESCs associated with pluripotency and differentiation. Since glycan molecules are candidates for sensitive markers, it is necessary to get more information about the stem cell-associated glycans for quality control in cell-based therapy.

2. Sequential multibranching on the common N-glycan core structure

N-glycans are branched chains. Sugar residues are attached to each other by α and β glycosidic linkages. They are generated from a common precursor through a common pathway, by sequential actions of glycosidases and glycosyltransferases (Figure 1).

The common precursor of N-glycans, consisting of 14 glycan units (2 N-acetylglucosamine (GlcNAc) units, 9 mannose units, and 3 glucose (Glc) units), is formed at the initial synthesis stage of all N-linked glycoproteins (Taylor and Drickamer, 2011; Varki et al., 2011; Bieberich, 2014). After transferring the precursor to the growing peptide, maturation reactions of the glycans start in the lumen of the rough endoplasmic reticulum and continue in the Golgi compartments. Glycosidases and glycosyltransferases successively modify the structure of the precursor. The differential actions of these enzymes cause the formation of a high mannose, hybrid, and complex types of N-linked oligosaccharides (Figure 1). All of these types have a common core structure consisting of 3 mannose residues and 2 GlcNAc residues. Two of the mannose residues are attached to the first mannose residue by 2 different linkages (α 1,6 and α 1,3), forming the initiation of 2 basic arms on the common core structure.

Specific N-acetylglucosaminyltransferases (GlcNAc-Ts, GnTs) (productions of MGAT genes) are responsible for the addition of new branches. In vertebrates, 7 different GnTs, indicated as GnT-I, -II, -III, -IV, -V, -VI and IX, and a fucosyltransferase (FUT8) have been determined to be involved in the initiation of branching in the complex N-glycan core structure (Taniguchi and Korekane, 2011; Takamatsu et al., 2013). GnTs catalyze the transfer of GlcNAc sugar from the active donor, uridine diphosphate GlcNAc, to specific positions on the core mannoses of N-glycans via a specific glycosidic linkage. Three, four, or more branches can be initiated by the activity of a specific GnT on the core structure (Chen et al., 2009).

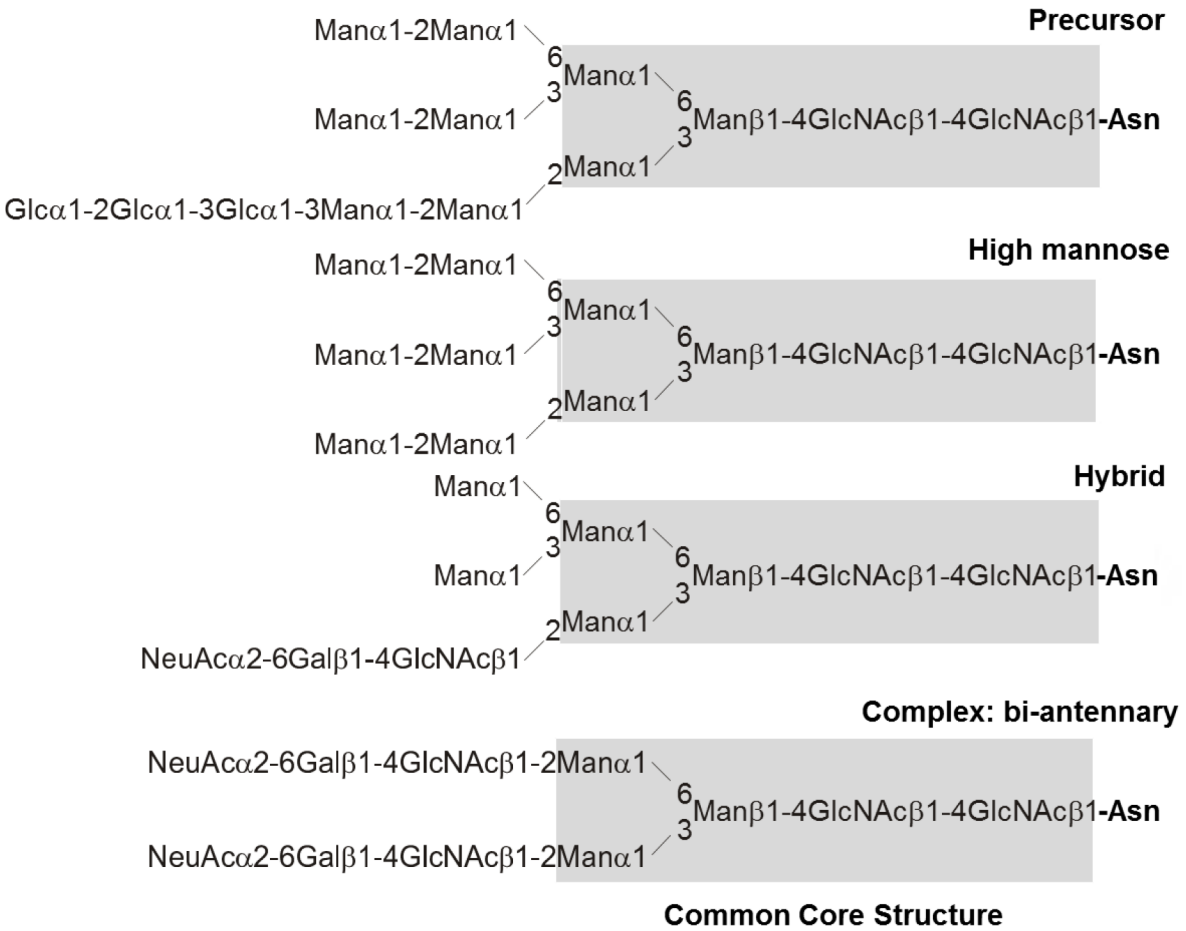

Figure 1. Precursor and basic types of N-glycans. The common core structure is shaded.

According to the sequential activity rules established by Brockhausen et al. (1988) for the GnTs, GnT-I and GnT-II are involved in initiating the synthesis of various branches of complex N-glycans. GnT-I acts before all the other GnTs and is required for the conversion of the high mannose type (with 5 mannose residues) to the hybrid and complex types (with 3 mannose residues) of N-glycans (Yip et al., 1997; Chen et al., 2002; Taniguchi and Korekane, 2011). It catalyzes the β 1,2 glycosidic linkage by transferring GlcNAc sugar to the mannose residue on the α 1-3 arm of the core structure. The α mannosidase II recognizes this structure, removes the 2 mannose residues from the α 1,6 arm, and a substrate for the GnT-II and GnT-III enzymes is formed.

GnT-II controls the conversion of hybrid-type to complex-type structures (D'Agostaro et al., 1995; Ye and Marth, 2004). GnT-II activity is a prerequisite (Zhang et al., 2000) for the GnT-IV, GnT-V, and GnT-IX activities, which are responsible for synthesis of the other branches on the common N-glycan core and are important for the conversion to cancer. GnT-II catalyzes the β 1,2 glycosidic linkage by adding a GlcNAc to the α 1,6 arm on the core; a 2-branched (biantennary) core structure is formed.

GnT-III catalyzes the formation of the β 1,4 glycosidic linkage by transferring a bisecting GlcNAc to the first mannose residue on the core (Figure 2). Various hybrid and complex N-glycans contain the bisecting GlcNAc. The presence of a bisecting GlcNAc prevents the catalytic activity of GnT-II, GnT-IV, GnT-V, and FUT 8 enzymes, which are responsible for branching of the core structure in vitro (Brockhausen et al., 1988; Isaji et al., 2010; Taniguchi and Korekane, 2011; Miwa et al., 2012; Xu et al., 2012).

3. Characteristic N-glycan structure-related pluripotency in hESCs/hiPSCs

A number of pluripotency biomarkers are currently used to characterize hESCs and hiPSCs. Stage-specific embryonic antigens SSEA-3 and SSEA-4 are known to be pluripotent glycosphingolipid glycan markers of hESCs and hiPSCs (Lanctot et al., 2007; Liang et al., 2010; Yanagisawa, 2010; Fujitani et al., 2013). A new and highly expressed surface marker, SSEA-5, was identified on the surface of

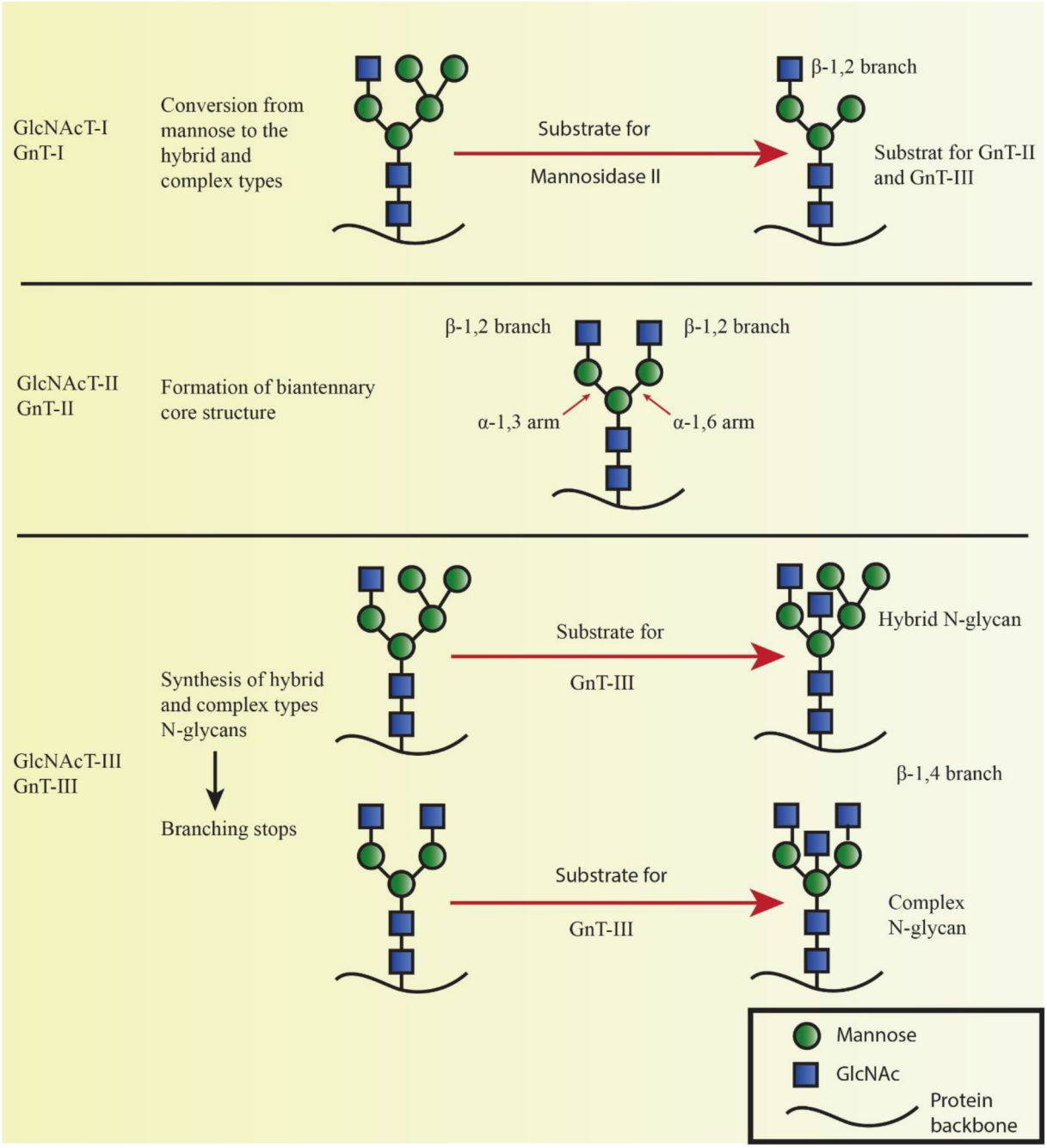

Figure 2. Initial branching on the common core structure of N-glycans, modified from Taniguchi and Korekane (2011) with permission.

human pluripotent stem cells (Tang et al., 2011). Tumor-rejection antigens Tra-1-60 and Tra-1-81 are widely used to characterize the hESCs (Wright and Andrews, 2009). They recognize the glycans specifically expressed on the type 1 lactosamine (Galβ1-3GlcNAc) structure (Fujitani et al., 2013).

In comparison with glycomic profiles, a lot of new and important information has been obtained using more sensitive analytical techniques.

High mannose-type and biantennary (fucosylated and sialylated) complex-type core structures are the major pluripotency-specific N-glycosylation features (Figure 3). High mannose-type N-glycan core structures include more than 5 mannose residues determined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and NMR spectroscopy (Satomaa et al., 2009). Even on the isolated cell membranes from hESCs, 9 mannose residues with

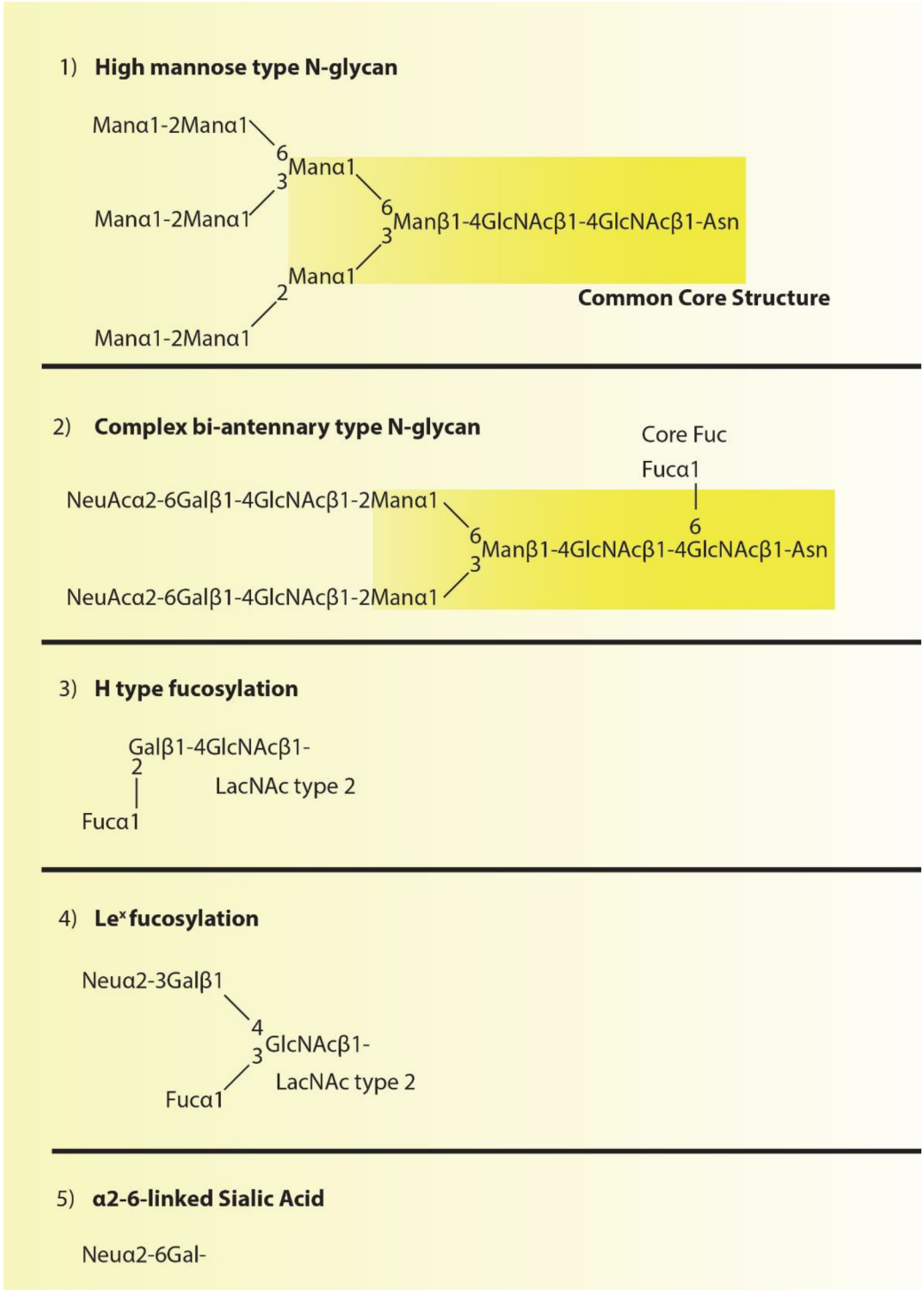

Figure 3. Pluripotency-relevant N-glycan features in hESCs.

intact terminal glucose residue were found using flow cytometry and immunocytochemistry (An et al., 2012). The results obtained from fluorescein-labeled lectin staining (Wearne et al., 2006), flow cytometry, and immunohistochemistry (Venable et al., 2005; Dodla et al., 2011) showed that hESCs expressed high percentages of high mannose glycans. In addition, upon induction of pluripotency, a significant increase occurred in the high mannose-type N-glycans, indicating the immature stage of N-glycoproteins (Hasehira et al., 2012; Hamouda et al.,

2014). High mannose-type N-glycans were also associated with the mesenchymal stem cell characteristic feature for human mesenchymal stem cells in a couple of studies (Hemmoranta et al., 2007; Heiskanen et al., 2009). On the contrary, in another study high mannose-type N-glycans were decreased in hESC and iPSC mixture (Tateno et al., 2011).

Biantennary complex-type core structures bear 2 LacNAc branches that are terminated with α 2,6 and α 2,3-linked sialic acids. A fucose residue is located subterminal to sialic acid. The MALDI-TOF MS spectrum (Satomaa et al., 2009), labeled lectins (Wearne et al., 2006; Dodla et al., 2011), and lectin microarray (Tateno et al., 2011) indicated the presence of these sugars. Since desialylated and defucosylated N-glycans of hESCs were sensitive to β 1,4- galactosidase but not to β 1,3 galactosidase digestion, it was suggested that the N-glycan antennae consist of type 2 N-acetyllactosamine (LacNAc, Gal β1-4 GlcNAc-) chains (Satomaa et al., 2009). However, the type 1 LacNAc (Galβ1-3GlcNAc) structure was suggested in particular as one of the characteristic feature of hiPSCs (Tateno et al., 2011; Hasehira et al., 2012). In addition to the N-glycans of hESCs carrying type 2 LacNAc (Satomaa et al., 2009), various type 1 LacNAc structures were also typical features of O-glycans and glycosphingolipids of pluripotent human cells (Natunen et al., 2011). This is possible by the activity of 3β-galactosyltransferase, which is also responsible for the elongation of oligosaccharide chains on both mucins and glycolipids (Sheares et al., 1982, 1983).

Complex terminal fucosylation is the other characteristic N-glycosylation feature of hESCs (Satomaa et al., 2009; Tateno et al, 2011; Hasehira et al., 2012). One terminal of the branches bears an α 1,2-linked fucose residue (Fuc α 1-2 Gal β 1-4 GlcNAc); the other terminal bears an α 1,3- or α 1,4-linked fucose residue, producing an Le^x structure (Gal β 1-4 (Fuc α 1-3) GlcNAc) (Satomaa et al., 2009). A recombinant peptide, rBC2LCN, recognizes and binds specifically to Fucα1-2Galβ1-3GlcNAc(GalNAc)-containing glycans. It has been suggested that it is an alternative to conventional pluripotent marker antibodies (Onuma et al., 2013). A core α 1,6-linked fucose residue, linked to the GlcNAc residue (which is directly attached to the asparagine residue of the protein), is found on the biantennary complex-type core structure. This type of α 1,6-linked fucosylation is the most abundant fucose linkage in hESC N-glycans (Satomaa et al., 2009).

The α 2,3- and α 2,6-linked sialic acids, with the α 2,6-linked one higher than the former, were seen on N-glycans of hESCs (Satomaa et al., 2009).

The linkage type of the sialic acid on N-linked glycans was dramatically changed from α 2,3 to α 2,6 upon induction of pluripotency (Tateno et al., 2011; Hasehira et al., 2012). A linkage shift may affect the biological functions of endogenous lectins. The binding activity of galectins is greatly diminished when LacNAc chains are capped with α 2,6-linked sialic acids (Iwaki et al., 2011; Hirabayashi et al., 2002). In summary, the high mannose-type and biantennary complex-type N-glycan core structures, type-2 LacNAc, terminal α 1,2 fucosylation, and α 2,6-linked sialic acids are all characteristic pluripotency features of the N-glycans in hESCs.

4. Changes in N-glycans during hESC differentiation

Characteristic N-glycan profiles of hESCs undergo some major changes during cell differentiation. Glycan structures on undifferentiated cells disappear and a new glycan profile occurs on embryoid bodies and on further differentiated cells. Comparison with fluorescein-labeled lectin images on embryoid bodies generated from BG01 hESCs (Wearne et al., 2006) and MALDI-TOF MS and proton NMR spectroscopic analyses on embryoid bodies formed from four Finish hESC lines (Satomaa et al., 2009) provided important information about glycan alteration during differentiation. Lectin microarrays obtained from four different somatic cell line-generating hiPSCs and from a number of hiPSCs clearly revealed these alterations (Tateno et al., 2011).

The low mannose-type, the hybrid-type, and the complex-type N-glycan core structures (Figure 2) were found more abundantly in differentiated cells (Satomaa et al., 2009). One of the early signs for differentiation-associated N-glycan changes is the increase in branching on the core structure of N-glycans. N-acetylglucosaminyltransferase-III (production of the MGAT gene) catalyzes the β 1,4 glycosidic linkage formation by transferring a bisecting GlcNAc to the first mannose residue on the core (Figure 2). N-glycan signals associated with differentiated cells (Satomaa et al., 2009; Tateno et al., 2011) and the distribution of PHA-E lectin ligands (Venable et al., 2005; Wearne et al., 2006) indicate this modification. The bisecting GlcNAc was found on various hybrid and complex N-glycans of healthy differentiated cells. The presence of bisecting GlcNAc prevents the subsequent processing and elongation of N-glycans, inhibiting the catalytic activity of the enzymes that are responsible for branching of the core structure (Brockhausen et al., 1988; Taniguchi and Korekane, 2011).

The other major differentiation stage-associated N-glycan signals are related to the multifucosylation of sialylated N-glycans, which are gradually decreased during hESC differentiation (Satomaa et al., 2009), where no α 1,2-linked terminal fucosylation appears (Wearne et al., 2006). Instead of terminal fucosylation, the α 1,6-linked core fucosylation increases (Tateno et al., 2011). PHA-L lectin signs were low in hiPSCs (Tateno et al., 2011), but they were significantly higher in differentiated human

neural progenitor and human mesenchymal progenitor cells (Dodla et al., 2011). This lectin is specific for the N-linked tri- and tetraantennary chains with Gal β1-4 GlcNAc on the β1-6 mannose branch.

In a transition embryoid body, the low mannose-type and the hybrid-type N-glycans were the characteristic structures (Satomaa et al., 2009). The hybrid-type N-glycans bear mainly the core fucose and their branches are terminated with both α 2,6- and α 2,3-linked sialic acids. N-acetylhexosamine compositions drastically increase during differentiation of hESCs into the embryoid body. The signal intensity of N-acetylhexosamine was found to be 3% for hESCs. An increase of 14% for the embryoid body and 22% for further differentiated cells was observed (Satomaa et al., 2009). The number of poly-N-acetyllactosaminyl chains on the core branches was associated with differentiated cells (Karaçalı et al., 2014). An increase in poly-N-acetyllactosaminyl repeats occurred in embryonic stem cells during differentiation (Wearne et al., 2006).

5. Conclusion

Extensive efforts have been made to understand the biological significance of the glycan parts of glycoconjugates. Pluripotent hESC and hiPSC N-glycans bear mainly high mannose-type and biantennary complex-type core structures. The α 1,2/1,3-linked terminal fucosylations and the α 2,6-linked sialic acids are the other characteristic features of pluripotency. For the effective use of stem cells in cell therapy, it is necessary to determine the pluripotency-associated glycans for isolation from other differentiated (healthy and tumor) cells.

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