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REVIEW

The cancer glycome: Carbohydrates as mediators of metastasis

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ABSTRACT

Glycosylation is a frequent post-translational modification which results in the addition of carbohydrate determinants, "glycans", to cell surface proteins and lipids. These glycan structures form the "glycome" and play an integral role in cell–cell and cell–matrix interactions through modulation of adhesion and cell trafficking.
Glycosylation is increasingly recognized as a modulator of the malignant phenotype of cancer cells, where the interaction between cells and the tumor micro-environment is altered to facilitate processes such as drug resistance and metastasis. Changes in glycosylation of cell surface adhesion molecules such as selectin ligands, integrins and mucins have been implicated in the pathogenesis of several solid and hematological malignancies, often with prognostic implications. In this review we focus on the functional significance of alterations in cancer cell glycosylation, in terms of cell adhesion, trafficking and the metastatic cascade and provide insights into the prognostic and therapeutic implications of recent findings in this fast-evolving niche.

1. Introduction

1.1. Physiological role of glycosylation

Glycosylation is a post-translational modification that occurs in the endoplasmic reticulum (ER) and results in the addition of carbohydrate motifs, "glycans", to proteins and lipids that are, in most cases, destined for the cell surface. The resultant "glycoprotein" or "glycolipid" structures at the cell surface form a carbohydrate rich layer which plays an integral role in the interaction of the cell with its surrounding environment. Of the more than 200 different types of protein PTMs, glycosylation occurs frequently and results in the addition of functional carbohydrate motifs to protein structures [1,2]. Glycans interact with carbohydrate binding proteins known as "lectins" that are specific for glycan moieties and are commonly used in purified form to study glycosylation *in-vitro*. One of the main functions of lectins in mammalian cells is to mediate cell–cell interactions and therefore interactions of glycans with their respective lectins have major implications for cell trafficking.

Glycosylation of a given protein is achieved through a complex series of post-translational enzymatic steps that lead to the formation of protein-bound glycans with specific and diverse biological functions. These carbohydrate side chains are capable of modulating the interaction of the protein with its environment influencing key factors such as protein half-life, solubility, binding activity and specificity. Proteins with the same amino acid sequence can possess different glycan structures, producing different glycoforms of the same protein. These glycoforms can differ in key properties such as stability, folding, localization and ligand specificity [3] with consequent implications for physiological processes, including protein folding and trafficking, cell–cell and cell–matrix interactions, cellular differentiation and the immune response [4–6]. Therefore, the glycosylation status of a protein can be used to differentiate protein glycoforms and molecular changes in glycosylation of proteins have been used to distinguish normal from disease states in humans [7,8]. Furthermore, as cell communication, adhesion, and signaling also play a major role in cancer, changes in glycosylation of surface proteins on malignant cells can alter interactions between cancer cells and their surrounding environment [6,9–11].

Glycosyltransferases are enzymes that regulate the process of glycosylation in humans where their action is dependent on the availability of precursor monosaccharide molecules and other parameters [12,13]. Glycosyltransferases, along with glycosidases, work to add and subtract monosaccharides to and from glycan structures, examples of these enzymes include sialyltransferases and fucosyltransferases, which are responsible for the addition of sialic acid and fucose moieties,

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respectively. The intracellular sites of action of these enzymes include the ER, golgi apparatus, cytosol and nucleus.

Two major types of glycosylation occur on proteins; 1) O-linked glycosylation refers to the addition of N-acetyl-galactosamine to serine or threonine residues by the enzyme UDP-N-acetyl-D-galactosamine transferase, this is then followed by the addition of other carbohydrates such as galactose, N-acetyl-D-glucosamine or sialic acid (Fig. 1).; 2) N-linked glycosylation occurs in the ER and refers to the process by which an oligosaccharide chain is enzymatically attached to the amide group of an asparagine in the consensus sequence Asn-X-Ser/Thr where X represents any residue except proline (Fig. 1). This sequence can be used to identify potential N-glycosylation sites in peptide sequences.

O-linked glycosylation also contributes to the production of proteoglycans by the addition of glycosaminoglycan (GAG) chains to a core protein. GAGs consist of repeating disaccharide units composed of an N-acetylated or N-sulfated hexosamine and either a uronic acid (glucuronic acid or iduronic acid) or a galactose. Examples of GAGs include hyaluronan, dermatan sulfate, keratan sulfate, chondroitin sulfate, heparin, and heparan sulfate. Heparan and chondroitin sulfate are linked to serine residues of core proteins by xylose and this process is mediated by a xylosyltransferase. Proteoglycans and their associated GAGs form essential components of the extracellular matrix where they function in cell adhesion *via* interactions between the complex carbohydrate motifs [14].

It is clear that alterations in gene expression and protein expression are not the sole factors responsible for phenotype determination in cancer cells, where not only the cell itself is affected, but also the microenvironmental components such as the extracellular matrix (ECM). The impact of post-translational modifications (PTMs) on proteins and lipids has identified a layer of complexity, beyond the amino acid sequence, which has the consequence of greatly altering the function and even the purpose of that protein in a given context. Although the protein sequence is governed by the relevant genomic code, many properties of functional cell surface proteins, and circulating glycoproteins, are governed by the modification of glycans and therefore consideration must be given to the glycosylation status of a protein when considering its activity within a biological system.

This rapidly developing field has provided new cancer biomarkers and potential targets recently in a variety of solid and hematological cancers [15–17]. In this review we focus on the enzymes involved in this process and the cell surface proteins that become modified as a result of their action, with an overall focus on the implications for cell trafficking and metastasis of cancer cells.

2. Carbohydrates and the cancer cell

2.1. Glycosylation and cancer

The normal process of glycosylation is disrupted during malignant transformation of cells [18,19]. These changes result in alterations in tumor cell surface glycans and therefore interactions with endogenous lectins are impacted, which influences the metastatic potential of the tumor cells. Complex carbohydrate structures that can be found attached to proteins and lipids on the surface of cancer cells have a major influence on their phenotype and the interactions that they have with the surrounding environment [20] (Fig. 2). In parallel with the changes in glycosylation, expression and levels of carbohydrate-binding proteins also change during malignant transformation leading to altered overall presentation of glycans and their cognate receptors, *i.e.*, lectins.

Alterations in glycosylation of malignant cells can take a variety of forms, including changes in the amount, linkage and acetylation of sialic acids, and changes in the branching of N-glycans mediated by glycosyltransferases, alterations in expression of glycosaminoglycans such as heparan sulfate, and altered glycosylation of mucins, which are heavily glycosylated epithelial-derived proteins known to be implicated in certain cancers [14]. Studies of the mechanisms by which alterations in glycans are able to bring about changes in cancer cell biology have been impeded by the complexity and heterogeneity of glycans, however recent advances in glycomics, including glycogenome analysis, HPLC, mass spectrometry and lectin profiling have facilitated comprehensive characterization of the glycome of several tissues [21].

The mechanisms by which glycosylation changes mediate tumor metastasis and invasion are mostly unknown, however roles of specific cell surface glycoproteins and their carbohydrate motifs have emerged and will be reviewed in the following sections.

2.2. Implications of glycosylation in cellular metastasis

Both solid and hematological malignancies begin the process of metastasis from the primary niche by escaping to the systemic circulation,

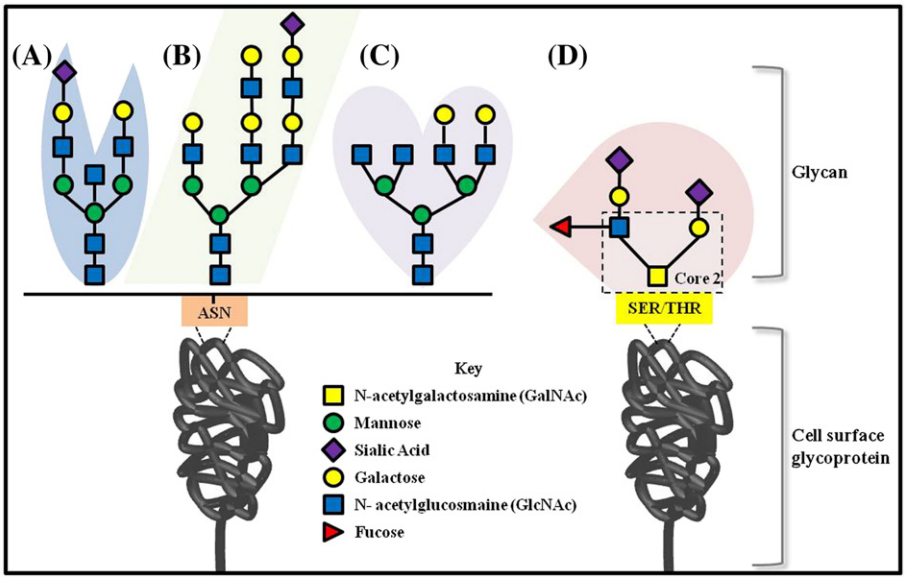

Fig. 1. O- and N-linked protein glycosylation. A–C: N-linked glycosylation; A — bisecting GlcNAc, B — Tri-antennary glycan, C — Tetra-antennary glycan. D: O-linked glycosylation, example shown is alpha 2, 3 sialylated glycan.

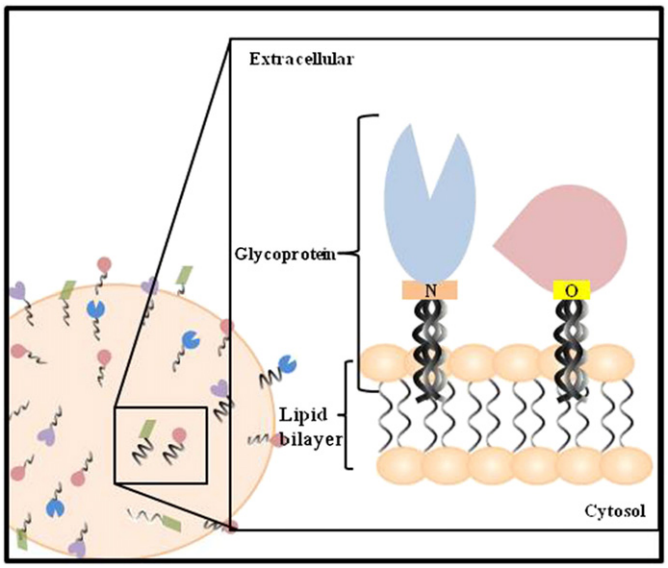

Fig. 2. Presentation of glycans on cell surface proteins. Representative image of N- and O-linked glycosylation on cell surface proteins, adding a layer of complexity composed of glycan moieties.

which is followed by extravasation to secondary sites and growth of distant metastatic lesions. Altered cell surface glycosylation has been implicated in this cascade where it supports tumorigenesis and metastasis [22,23]. During the process of transformation, from a normal cell to its malignant counterpart, cells acquire several mutations in proto-oncogenes and tumor suppressor genes giving rise to an altered phenotype. However, changes also occur at the cell surface, which alter the interaction of cancer cells with the surrounding environment, and facilitate participation in the multi-step process of metastasis. Alteration of tumor-cell-surface glycosylation changes the extracellular "velcro" layer and results in differential adhesive and invasive properties of these cells. Cancer cells in the circulation extravasate into tissues and form new metastatic niches using mechanisms that normally function to recruit leucocytes to sites of inflammation and injury [24]. Adhesion molecules expressed on the surface of cancer cells play a crucial role in metastasis and the ability of cancer cells to metastasize is largely determined by their ability to interact with endothelium, which is mediated, at the initial phases, by integrins and selectins. Selectins are a family of three transmembrane adhesion molecules that are expressed on the surface of leukocytes (L-selectin), platelets (P-selectin) and vascular endothelial cells (E and P-selectin) [25]. Selectins are expressed on endothelial cells and interact with their ligands on cancer cells to play an important role in initiating the metastatic process by regulating the tethering and rolling of cancer cells to the vascular endothelium, a pre-requisite for subsequent transendothelial migration across the vessel wall (Fig. 3). The interaction of selectins with their ligands is greatly influenced by glycosylation and some glycans such as sialyl Lewis X (SLe^X) and sialyl Lewis A (SLe^a) play a critical role in E-selectin ligand function. Integrins are large transmembrane glycoproteins that serve as cell–cell adhesion molecules and are responsible for mediating the interaction of cells with extracellular matrix (ECM) components such as collagens, fibronectin and laminins. We focus in our review on four important differentially glycosylated proteins in cancer; selectin ligands, integrins, mucins and galectins, and on the enzymes mediating these changes with a view to implications for cell trafficking and metastasis.

3. Targets of glycan modifications in cancer

3.1. Selectins

As previously mentioned, selectins are vascular cell adhesion molecules which mediate adhesion of leukocytes and platelets with the endothelium. There are three members of the selectin family: P-, E-, and L-selectins. P-selectin is present in the storage granules of platelets (α-granules) and endothelial cells (Weibel–Palade bodies), and rapidly translocates to the cell surface upon activation [26]. L-selectin is expressed on the surface of almost all leukocytes. The physiological functions of selectins are well described in processes of immune response inflammation, cell trafficking, and hemostasis [27] largely through the study of specific selectin knockout mouse models.

L-Selectin mediates fast rolling of leukocytes on endothelium while P- and E-selectin support the rolling at lower velocities within the vasculature [27]. The initial steps in cell migration involve tethering and

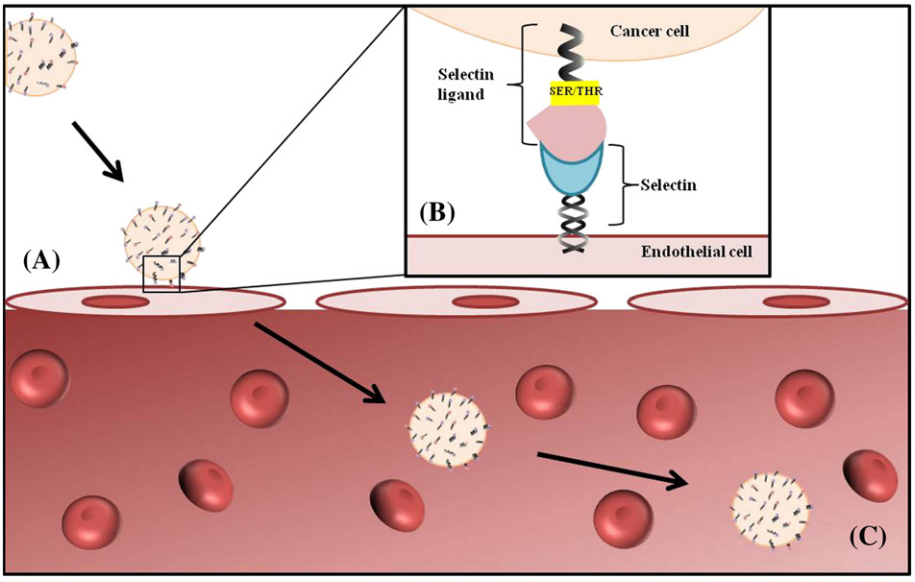

Fig. 3. Selectins interacting with glycosylated ligands at the surface of cancer cells during the process of hematogenous metastasis. A) Cancer cell initial interaction with endothelial cells followed by tethering and rolling of the cell on endothelium. B) Critical interaction between the selectin ligand of a cancer cell and selectin on an endothelial cell mediated by sialylated glycans on selectin ligands. C) Transendothelial migration of cancer cell allowing entry into the blood stream and hematogenous metastasis.

rolling of cells on the vascular endothelium, which is mediated by the interaction of selectins on the endothelial surface and their carbohydrate ligands. Structurally, selectin ligands consist of distinct glycan structures, which incorporate the terminal core tetrasaccharide structure SLe^x and SLe^a on a protein backbone. Selectins can bind to various classes of molecules including mucins, sulfated glycolipids and glycosaminoglycans, and most of these molecules are capable of acting as functional selectin ligands *in-vivo* [26].

During the hematogenous phase of metastasis, selectin ligand-expressing tumor cells commonly encounter selectins, present on leukocytes, platelets and endothelium in the circulation [28,29]. Following initial tethering rolling leukocytes are activated by binding to selectins and by chemoattractants like CXCR4/SDF-1 the presence of a chemotactic signal outside the venule induces leukocytes to extravasate.

Selectin-ligand glycosylation is modified by several glycosyltransferases that cooperate to form functional selectin ligands. These include fucosyltransferases FucT-VII and FucT-IV [30,31], core 2 β1-6-N-acetylglucosaminyltransferase-1, [32,33] and several of the sialyltransferases [34,35]. SLe(x) is a tetrasaccharide carbohydrate ligand that forms an essential component of selectin ligands as mentioned previously, [35] and mediates the migration of healthy leukocytes to sites of injury where they perform necessary immune functions [36]. Tumor cells are able to hijack this normal mechanism of cellular trafficking which enables them to gain efficient metastatic potential. Higher levels of membrane associated SLe^x on cancer cells promotes metastasis via this mechanism [37]. SLe^x can be found at the non-reducing terminus of a glycan chain where structurally it contains a Galβ1-4GlcNAc backbone. Attached to the Gal is a α2-3NeuAc and the GlcNAc has a α1-3Fuc attached. SLe^x synthesis is initiated by adding α2-3NeuAc to the Gal of N-acetyllactosamine; this reaction is catalyzed by βgal:α2-3 sialyltransferases, of which there are several in humans [38]. Following this, α1,3/4-fucosyltransferases catalyze the addition of α1-3Fuc to the GlcNAc [39,40]. Once in place on a selectin ligand, SLe^x determines binding specificity and directs functionality of various selectin ligands; therefore alterations in this glycan structure have major implications for cell trafficking and metastasis.

P-Selectin glycoprotein ligand-1 (PSGL-1) is the predominant physiologic ligand for P-selectin and L-selectin, but when modified by HECA-452 reactive glycans (CLA) it can also serve as an E-selectin ligand [41,42]. PSGL-1 plays a role in leukocyte trafficking and PSGL-1 mediated rolling is a pre-requisite for integrin mediated firm adhesion and PSGL-1 up-regulation in immune cells may be a mechanism of enhanced migration [43]. Posttranslational modifications of PSGL-1 are important for optimal selectin binding [44]; to bind to P-selectin, PSGL-1 requires an α 2,3-sialylated and α 1,3-fucosylated core 2 O-glycan attached to a specific N-terminal threonine with tyrosine sulfation near the N-terminus optimizing the binding to P-selectin [44–46]. To bind to E-selectin, PSGL-1 requires core 2 α 1,3-fucosylated and α 2,3-sialylated O-glycans, indicating a crucial role for ST3GAL-VI in the function of PSGL-1 as both an E and a P-selectin ligand [44,47]. PSGL-1 has previously been shown to regulate the adhesion and homing of MM cells to the bone marrow niche and regulates proliferation and development of drug resistance in MM cells [48]. Furthermore PSGL-1 is critical for macrophage-mediated MM cell drug resistance [49], both of these studies outline an important role for PSGL-1 in MM biology.

We believe that ST3GAL6-VI may be important in the generation of E-selectin ligands, which mediate homing and retention of MM cells in the bone marrow via interaction with E-selectin and hypothesize that overcoming the interaction of E-selectin and its ligands could be a useful chemosensitizing strategy in MM and AML. Both normal hematopoietic stem cells and cancer stem cells are known to express E-selectin ligands [50]. Recent data demonstrates that selectins and their ligands are required for homing and engraftment of $BCR\text{-}ABL^+$ leukemic stem cells in the bone marrow niche and adhesion of colon carcinoma cells to E selectin activates survival pathways, such as NFκB [51,52].

3.2. Integrins

As mentioned, integrins are large complex transmembrane glycoproteins that act as cell adhesion molecules. The integrin family is made up of 24 members comprises 24 members, that consist of a combination of 1 of 18 α- and 1 of 8 β-subunits [53]. Integrins directly bind components of the extracellular matrix such as laminin, fibronectin and collagen and themselves convey signals downstream, following binding to extracellular ligands. This occurs via a variety of cell signaling molecules such as focal adhesion kinase (FAK) and Src via activation of kinases, GTPases and the Ras/Rho pathways [54]. Through these mechanisms integrins can modulate cell adhesion, migration and proliferation and have therefore been extensively investigated in cancer. The ubiquitous presence of integrins on tumor cells, blood components, vasculature, and stromal cells suggests that integrins contribute to the metastatic cascade. In cancer, integrins display altered branching of N-glycans, mediated by N-acetylglucosaminyltransferase III (GnT-III) and N-acetylglucosaminyltransferase V (GnT-V). Typically integrin glycosylation by GnT III inhibits cell migration while integrins glycosylated by GnT-V promote cell migration [55]. In colorectal cancer, alterations to the N-glycan branching of integrins have been shown to contribute to a more invasive phenotype [56]. The modification of integrin-associated glycans can also be mediated by sialyltransferases, which has been demonstrated in pancreatic cancer where ST3GAL-III transfected cells exhibited higher SLe^x and lower α2,6-sialic acid content on the glycans of their α2β1 integrin molecules and higher adhesive potential [57]. Therefore, it is clear that altered integrin glycosylation has major implications for cell adhesion and metastatic potential, making this an important focus of glycosylation research in cancer.

3.3. Mucins

Mucins are large glycoproteins with a “bottle-brush” like conformation, which carry many clustered glycosylated serines and threonines in tandem repeat regions. The human mucin family (MUC) consists of 21 mucins (MUC1 — 21) that are further classified as secreted or transmembrane mucins and can be found expressed on normal and malignant epithelial cells [58,59]. In normal tissues, mucins serve to protect cells from the microenvironment by formation of a protective layer [37]. Mucins are aberrantly expressed in many cancers where they display differential patterns of glycosylation and many cancer-associated mucins (and their glycans) serve as circulating biomarkers with clinical utility [60]. Specific O-glycans that are associated with mucins in cancer include the carbohydrate antigens Tn (GalNAcα1-O-Ser/Thr), STn (NeuAcα2-6GalNAcα1-O-Ser/Thr), and T (Galβ1-3GalNAcα1-O-Ser/Thr) [61,62]. Aberrant glycosylation of mucins is a common feature of all adenocarcinomas and tumor cells are known to express mucins that are associated with the epithelium from which they are derived, alongside new cancer-associated mucin core structures and glycan structures [63]. Mucins are generally found to be expressed on the apical domain of epithelium with soluble mucins being secreted into the lumen. During malignant transformation there is disarray of correct mucin expression on endothelial cells which allows soluble mucins to enter the extracellular space and circulation [14]. Due to their circulating nature and because they can be detected by monoclonal antibodies, mucins have been proposed as prognostic and diagnostic markers in several cancers including the well known markers CA-125 and CA19-9. In cancers of epithelial origin in particular, mucins appear to be the major carriers of altered glycosylation [14].

It is likely that cancer cells alter their mucin expression in order to allow them to interact appropriately with the tumor microenvironment and enhance survival signaling. SLe^x can be considered a mucin glycan as it is frequently associated with mucins and, as mentioned, it has been demonstrated in colonic carcinoma cell lines that 5-Aza-2′-deoxycytidine (5-Aza) treatment enhances SLe^x production on the mucin MUC1 by inducing the expression of ST3 β-galactoside α-2,3-

sialyltransferase 6 (ST3GAL-VI) [64]. 5-Aza is an inhibitor of DNA methyltransferase which leads to DNA hypomethylation, weakening the effects of natural gene silencing mechanisms. Following treatment, the colonic carcinoma cell line also showed a corresponding increase in the adherence of these cells to E-selectin under dynamic flow conditions. Knockdown of ST3GAL-VI in these cells reduced the level of SLe^x without affecting MUC1 expression. This points towards hypomethylation as a mechanism of regulation of ST3GAL-VI and consequently SLe^x expression on mucins [64].

In pancreatic cancer, differential glycosylation of mucins MUC1 and MUC4 is apparent as the disease progresses from early adenocarcinoma to the metastatic state and MUC1 has been shown to induce multidrug resistance gene expression [65–67]. Tn and STn are highly expressed in the pancreatic cancer tissue however, STn antigen on MUC1 is associated with the malignant state whereas Tn on MUC1 is observed in normal pancreatic ductal cells [67]. The presence of high levels of Tn and STn structures on cancer cells is well described and may in part be due to mutations in the Cosmc protein [68,69], the core 1 synthase enzyme or as previously stated any of the enzymes involved in O-glycan extension. Cosmc is a chaperone protein that is necessary for core 1 activity and consequently the extension of Tn glycan into core 1 or core 2 structures, including the T antigen [67]. It has recently been demonstrated that glycan elongation beyond the mucin-associated Tn antigen in pancreatic and breast cancer protects cancer cells from immune mediated killing by natural killer (NK) and T-cells, indicating that alterations in glycosylation can mediate cancer cell immune escape [70]. It has previously been shown that the extracellular portion of MUC1 binds to protumorigenic factors such as Galectin-3 [71]; this interaction likely influences downstream signaling events as the cytoplasmic tail of MUC1 interacts with a number of receptor tyrosine kinases. MUC4 associates with the ErbB2 receptor to affect proliferation, apoptosis and epithelial mesenchymal transition (EMT) in cancer cells [72–74].

Therefore, it stands that differential glycosylation of mucins has a broad array of effects on the cancer cell glycome itself but also on immune escape of cancer cells, survival and proliferation.

3.4. Galectins

Galectins are a family of 15 immunoregulatory lectins which bind to galactose that is either β1,3 or β1,4 linked to N-acetylglucosamine [63]. Galectins are soluble proteins with both intracellular and extracellular functions and are expressed by a wide variety of cells including epithelial and immune cells where they are bound to proteins by both N-linked and O-linked glycosylation. They have a broad range of function including the mediation of cell–cell interaction, cell–matrix adhesion, apoptosis regulation and supression of T-cell receptor activation [75,76].

Several studies point towards a role for galectins in regulating cancer cell functions such as adhesion, invasion and metastasis [77,78]. Galectin-3 has been extensively implicated in several cancers where its presence on cancer cells themselves or on endothelial cells can help to promote adhesion and metastasis [79,80]. In colon cancer cells, homotypic interactions between Galectin-3 and MUC1, both present on the cell surface, increase the survival of tumor cells and promote embolism formation and dissemination of tumor cells [79]. Similar homotypic interactions, mediated by Galectin-3, are also observed in highly metastatic breast cancer cells [80]. These and other studies highlight the interplay of cancer glycome components in promoting metastasis through glycan specific interactions. Galectin-3 has also been found to be highly expressed in Diffuse Large B Cell Lymphoma (DLBCL) [81] where the expression has been linked to a poor outcome for patients [82]. Galectin-3 is also aberrantly expressed in many other types of cancer where it has been shown to mediate apoptosis, possibly via BCL-2 and in leukemia cells increased Galectin-3 facilitates survival via stabilization anti-apoptotic BCL-2 family members [83,84]. Furthermore, the gene encoding Galectin-3, LGALS3, is upregulated in acute myeloid leukemia where it has been shown to be independently associated with an unfavorable outcome in these patients [85].

Galectin-1 has also been reported to promote tumor growth by inducing apoptosis of tumor responsive activated T-cells following glycan specific binding to CD45 or CD43 on T-cells [63,86,87].

Galectins have been targeted in cancer using modified citrus pectins (MCP) which are complex carbohydrates capable of combining with the carbohydrate-binding domain of Galectin-3 [88]. In multiple myeloma one of these compounds was able to induce apoptosis in various multiple myeloma cell lines, including those resistant to dexamethasone, melphalan, or doxorubicin. Interestingly this compound was able to overcome the growth advantage conferred by antiapoptotic protein Bcl-2, heat shock protein-27, and nuclear factor-κB, and blocks vascular endothelial growth factor-induced migration of multiple myeloma cells [89]. This same compound has recently been found capable of removing cell-surface Galectin-3 from CD45 rendering DLBCL cells susceptible to chemotherapeutic agents. This was shown to be regulated by C2GnT-1 glycosyltransferase [90]. The need for the development of Galectins, particularly Galectin-3 is apparent and efforts to address this unmet need in cancer are underway [91].

4. Glycosyltransferases: mediators of carbohydrate modifications in cancer

Glycosyltransferases are a large and diverse family of enzymes that are responsible for the assembly of monosaccharide moieties into linear and branched glycan chains. These enzymes tend to act sequentially so that the product of one enzyme prepares its acceptor as the substrate of the next enzyme in the process. Glycosyltransferases are specific for the type of linkage (α or β), and the linkage position of the glycoside bond formed [e.g. $\alpha(1 \rightarrow 3)$ or $\beta(1 \rightarrow 4)$]. Glycosyltransferases were initially considered to be specific for a single glycosyl donor and acceptor, which led to the "one enzyme-one linkage" concept [92]. Subsequent observations have refuted the theory of absolute enzymatic specificity by describing the transfer of analogs of some nucleoside mono- or diphosphate sugar donors and it is now clear that some glycans may be assembled by the action of any one of a number of highly related transferases [93]. Sugar nucleotide donors for glycosyltransferases in humans are: UDP-glucose, UDP-galactose,UDP-GlcNAc, UDP-GalNAc, UDP-xylose, UDP-glucuronic acid, GDP-mannose, GDP-fucose, and CMP-sialic acid.

As mentioned previously N-linked glycosylation begins in the ER with the synthesis of dolichol-linked GlcNAc sugar which is then constructed into a precursor oligosaccharide and extended to a precursor glycan by the additions of 2 GlcNAc, 9 mannose and 3 glucose molecules (Dolichol-$GlcNAc_2$-Man_9-Glc_3). The precursor glycan is transported to a protein in the lumen of the ER where an oligosaccharyltransferase recognizes the consensus sequence (Asn-X-Ser or Asn-X-Thr) of the polypeptide acceptor. The glycan is further processed in the ER by glycosidases I and II and a series of mannosidases. Following this glycosyltransferases add sugar residues to the core glycan structure, giving rise to the three main types of N-glycans (Fig. 1).

While N-glycosylation is the most common glycosidic linkage, O-glycosylation also plays a key role in cancer biology, as previously highlighted. Mucin synthesis requires O-glycosylation it is also critical for the formation of proteoglycan core proteins that are important components of the extracellular matrix. The linkage mechanism involved in O-glycosylation is not as complex as that of N-glycosylation and O-glycosylation also differs in that glycans are added one-at-a-time to serine or threonine residues. Proteins are O-glycosylated in the Golgi by N-acetylgalactosamine (GalNAc) transferase, which transfers a single GalNAc residue to the β-OH group of serine or threonine. Proteins can also undergo O-glycosylation with GlcNAc, fucose, xylose, galactose or mannose, depending on the cell and species. As with N-glycosylation, sugar nucleotides serve as monosaccharide donors for

O-glycosylation which then continues as various sugars are added to the growing glycan chain [13,94].

Common categories of changes in N and O-linked glycosylation occurring in cancer and specific glycosyltransferases involved are covered in the below sections.

4.1. Altered branching and truncation of glycans in cancer

An important example of the action of glycosyltransferases in cancer is in relation to the branching of N-glycans on the surface of cancer cells. An increase in the size of N-glycans on the surface of cancer cells has been attributed to an increase in β1-6 branching of N-glycans that results from enhanced expression of GnT-V. This glycosyltransferase is coded on MGAT5 and this gene is induced in cancer transformation in several cancers, including hepatocellular cancer (HCC) [95]. Cell lines with increased GnT-V expression show an increased frequency of metastasis in animal models and when this enzyme is lost cells lose this metastatic phenotype [96,97]. Breast cancer tumor development and metastasis has been shown to be significantly less in MGAT-5 deficient mice and the absence of MGAT-5 has been shown to induce the activation of phosphatidylinositol 3 kinase (PI3K) [98]. Integrins were identified in this study as specific major target glycoproteins of MGAT-5. MGAT-5 is currently being evaluated as a potential glyco-target and a compound that partially blocks MGAT-5, by diverting the biosynthesis pathway upstream of the enzyme, has shown activity against breast cancer in mice [98]. Furthermore, Ma and colleagues demonstrated that inhibiting MGAT-5 expression in a murine breast cancer cell line significantly reduced breast cancer cell proliferation following a reduction in complex surface N-glycans which translated to reduced tumor progression *in-vitro* and *in-vivo* [99]. Although it has been demonstrated in several models that MGAT-5 knockout can reduce tumor growth and metastasis *in-vitro* and *in-vivo* the mechanism has not been fully defined. Morgan et al. proposed that MGAT5-5 mediated N-glycosylation negatively regulates Th1 cytokine production [100].

There is also some evidence that MGAT-5 can affect integrin stability directly through modification of glycans. This was demonstrated in a study by Wang et al. [101] that showed attenuation of the number of β1-6 GlcNAc branching structures on β1 integrin in MGAT-5-inactive-mutant transfected hepatocellular cancer cell lines. Interestingly, there was a decreased β1 integrin expression in the inactive-mutant transfected cell line despite no significant change in the mRNA level, suggesting that the presence of β1-6 GlcNAc branching contributed to the expression of a more mature and stably expressed form of β1 integrin and as a functional consequence was more effective in promoting cell migration to fibronectin. MGAT-5 may also regulate expression of cytokine receptors as reported by Partridge et al. [102] who demonstrated that the expression of MGAT-5 sensitized mouse cells to multiple cytokines through promoting the substitution of N-glycan with N-acetyllactosamine, the preferred ligand for Galectin-3. In this case MGAT-5 was shown to be rate limiting factor for cytokine signaling and consequently for epithelial mesenchymal transition, cell motility and tumor metastasis [102].

Since certain glycan signatures can be linked to malignancy and metastasis, efforts have been made to assess the glycan profile of drug and chemotherapy resistant cancer cells. HT-29 colon cancer cells have been shown to have higher levels of α 2,3 and α 2,6 sialylated structures when methotrexate resistance develops [103]. Various techniques have been employed to evaluate the cell surface glycan profile of therapy resistant cancer cells ranging from lectin binding studies to mass spectrometry. Down regulation of MGAT-5 has been shown to enhance chemosensitivity in breast cancer [99] and deglycosylation with PNGase-F which cleaves N-linked glycans from the proximal GlcNAc residue also produced a similar effect. The glycan products of MGAT-5 act as tumor-associated glycan markers and they are commonly increased in a variety of malignancies where levels have been shown to correlate with disease progression [104–106]. Also, β1-6 branched oligosaccharides have been shown to be increased in breast cancer, as demonstrated by phaseolus vulgaris lectin-L (PHA-L) binding. PHA-L recognizes and binds specifically to terminal galactose, N-acetylglucosamine and mannose residues of complex glycans on mammalian glycoproteins, and in this study was shown to be predictive of a worse outcome [107].

Altered truncation of glycans in cancer is not restricted to N-linked glycans. Truncation O-linked glycans such as T, Tn, sT, Tn on mucins of epithelial cancers, such as breast ovarian and colorectal cancer, has also been noted [108–111]. Increased expression of sTn antigen in these cancers has been proposed as a biomarker due to its association with inferior outcomes in these cancers [112,113].

4.2. Sialyltransferases

Sialyltransferases are enzymes that transfer sialic acid from the activated cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NeuAc) to terminal positions on sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins (Fig. 1). Sialyltransferases belong to glycosyltransferase family with 29 members which comprises enzymes that can be classified according to the Carbohydrate-Active enZYmes system (http://www.cazy.org) sialyltransferase; β-galactoside α-2,6-sialyltransferase; α-N-acetylgalactosaminide α-2,6-sialyltransferase; β-galactoside α-2,3-sialyltransferase; N-acetyllactosaminide α-2,3-sialyltransferase; (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase; α-N-acetyl-neuraminide α-2,8-sialyltransferase; lactosylceramide α-2,3-sialyltransferase.

Sialyltransferases have been shown to be aberrantly expressed in several cancer models, most prominently reported in the literature are ST3GAL-I and ST3GAL-IV and ST3GAL-VI [114–116], additionally, our group has recently reported a role for ST3GAL-VI in migration and trafficking of multiple myeloma cells *in-vitro* and *in-vivo* [117]. Moreover, we showed that high levels of expression of ST3GAL-VI were independently associated with reduced survival in patients treated on the MRC Myeloma XI study [117]. As previously mentioned, aberrant glycosylation is a recurring theme in breast cancer and consequently O-linked glycosylation has been extensively studied in this disease and sialylated core 1 chains are reported to be expressed at higher levels on breast cancer cells than in their normal mammary counterparts, where core 2 based O-linked glycans predominate [114]. This has been attributed to the over-expression of ST3GAL-I [118,119]. ST3GAL-I is upregulated by COX-2 in breast cancer and this is mediated *via* PGE2 [114]. COX-2 has been implicated in the induction of several malignancies [120,121] where it may also be exerting its effect *via* alterations in glycosylation. A previous study by this group demonstrated that spontaneous Polyoma virus middle T antigen (PyMT) induced mammary tumors developed earlier when human ST3GAL-I was expressed as a transgene driven by the MUC1 promoter to ensure expression of the sialyltransferase in the mammary gland [122].

One mechanism by which sialyltransferases may contribute to an enhanced metastatic phenotype in cancer cells is *via* the generation of SLe^x, which is known to serve as a selectin ligand and therefore has implications in the interaction of endothelial selectins with their ligands on the surface of cancer cells. Treatment of neutrophils with sialidases has produced evidence that sialic acids may play an important role in selectin ligand function [34,123]. There are six different sialyltransferases present in mammals that have the ability to generate α 2–3 sialic acid linkages on glycoproteins and glycolipids, all of which could therefore theoretically contribute to the generation of SLe^x and be implicated in the malignant phenotype of cells [124]. Sperandio and colleagues have demonstrated that ST3GAL-IV-deficient mice have a defect in selectin ligand function *in-vivo*, including a mild reduction in E-selectin-dependent rolling, an increase in E-selectin-dependent rolling velocity, and a decrease in L-selectin-dependent rolling during inflammation, which relies on P-selectin glycoprotein ligand-1 PSGL-1, the predominant selectin ligand expressed on leukocytes [34,35]. It

was noted that ST3GAL-IV deficiency alone does not account for the full contribution of sialyltransferases to selectin ligand synthesis and *in-vivo* studies were carried out to further evaluate this. ST3GAL-VI deficient and ST3GAL-IV/VI double-deficient mice were found to have a deficiency in P-selectin mediated leukocyte rolling in an *ex-vivo* chamber system. This was seen in the leukocytes from ST3GAL-VI deficient mice and was more pronounced in the double deficient mice where it was equivalent to that of sialidase treated leukocytes. The most pronounced effect of ST3GAL-VI function was apparent in P-selectin ligand formation. Neutrophil recruitment into the inflamed peritoneal cavity and lymphocyte homing to secondary lymphoid organs were impaired in ST3GAL-VI-null mice and more severely in double-deficient mice [124]. This provided the first evidence of a coordinated role for these sialyltransferases in selectin ligand synthesis. Through a coordinated process these enzymes work to generate SLe^x or its sulfated form, 6-sulfo-SLe^x on glycoproteins or glycolipids with binding activity to selectins [125]. The absence of one or more of these enzymes can alter leukocyte rolling with implications for cancer cell adhesion, homing and metastasis. Our group has demonstrated that in multiple myeloma, a cancer demonstrating widespread cell trafficking at diagnosis, knockdown of ST3GAL-VI, which is expressed at high levels at the mRNA level in cell lines and patients, results in reduced adhesion and transendothelial migration of multiple myeloma cells *in-vitro* alongside a reduction in bone marrow homing *in-vivo* resulting in prolonged survival of xenograft mice [117]. This provides evidence of functional consequences of alterations in cell surface glycosylation in multiple myeloma cells, where α 2,3 sialylation may participate in selectin ligand formation and therefore impact cellular trafficking and metastasis.

The enhanced expression of carbohydrate ligands such as SLe^x is well established in several cancer models but the molecular mechanisms that lead to this are not well understood. Evidence is accumulating indicating an interaction between epigenomics and alterations in the human glycome [126], for example the accumulation of SLe^x in colon cancer cells may be as a result of DNA hypomethylation [64]. It is likely however that this cannot explain the diversity of changes seen in the carbohydrate determinants on cancer-associated ligands. Another interesting proposed mechanism relates to alterations in sugar transportation and intermediate carbohydrate metabolism. Cancer cells exhibit a metabolic shift from oxidative to anerobic glycolysis, this is known as the Warburg effect, which corresponds to increased gene expression of sugar transporters and glycolytic enzymes in cancer cells. These changes have been recently linked to induction of genes related to the expression of SLe^x in cancer [127]. This includes ST3GAL-I and Fuc-T VII, which are induced when colon cancer cells are grown under hypoxic conditions; interestingly this is believed to be mediated by hypoxia inducible factor (HIF) [128]. This leads to higher expression of SLe^x and SLe^a on cancer cells and is likely to at least partially explain the increased SLe^x determinant expression seen in some cancers which was accompanied, in this study, by a concomitant increase in E-selectin binding activity. This process refers to the "neosynthesis" hypothesis related to the mechanism of enhanced expression of carbohydrate determinants of selectin ligands in cancers [129,130]. This was further examined in a study looking at the association between SLe^x and SLe^a expression on colon cancer cells and EMT. The induction of EMT was shown to increase SLe^x and SLe^a expression and enhance E-selectin binding. In this study transcript levels of ST3GA-I/III/IV and FUT-III were significantly elevated and found to be regulated by c-Myc. This study outlines the role of SLe^x and SLe^a expression in mediating selectin binding during EMT [131].

4.3. Fucosyltransferases

Fucosyltransferases are a family of enzymes that transfer L-fucose sugar from a GDP-fucose (guanosine diphosphate-fucose) donor substrate to an acceptor substrate, such as core GlcNAc (N-acetylglucosamine) sugar, in the case of N-linked glycosylation, or to a protein, in the case of O-linked glycosylation produced by O-fucosyltransferase. Along with having sialic acid as its terminal sugar SLe^x also has fucose which is regulated by FUTI-VII and FUT-IX [132,133]. So it stands, as for sialic acid, that manipulation of the fucose may influence selectin ligand synthesis and interactions in a similar manner to that of sialic acid; indeed this has been shown to be the case in several cancer models. In colon cancer SLe^x expression is not only regulated by sialyltransferases but has also been shown to be regulated by the fucosyltransferases such as FUT-VI and FUTIII. Inhibition of FUT-III in colon cancer inhibits selectin mediated adhesion and metastasis [134] and Fuc-TVI knockdown is associated with a reduction in SLe^x expression in colon cancer cell lines [135]. Zandberg and colleagues adopted an interesting metabolic engineering strategy to inhibit the biosynthesis of SLe^x in cancer cells using peracetylated 5-thio-L-fucose. Blockade of fucosyltransferases led to functionally significant impairments in SLe^x levels and selectin mediated adhesion [136]. The abovementioned fucosylation relates to the addition of fucose residues to the N- and O-linked terminus in an α2,3 and/or 4 linkage pattern; however the fucosylation of the core structure of N-glycans has also been reported to be altered in cancer cells [133]. Core fucosylation is carried out by FUT-VIII and has been found to be elevated in breast, colon, ovarian and lung cancer [119,137–141]. The importance of the fucosylation in the ability of cancer cells to migrate and metastasize therefore appears to be mainly related to their role in the synthesis of SLe^x and the implications of these changes for selectin ligands, targeting altered fucosylation in cancer cells is therefore an attractive therapeutic strategy given the importance of this process in cell adhesion and trafficking.

5. Mechanisms of regulation of glycosylation changes in cancer

Although glycans have been shown to be extensively altered in cancer, the mechanisms of regulation that govern the expression of the implicated genes are not well understood. It is likely that the genetic landscape of glycomics is not regulated by any one process but instead is an interplay of many factors, made more complex in the malignant state. However progress in this area is being made and evidence is accumulating that these genes may be altered by hypoxic conditions in the local microenvironment or may also be regulated by methylation.

5.1. Hypoxia and glycan expression

Under the poorly oxygenated conditions found in locally advanced tumors, hypoxia-resistant cancer cells survive by acquiring hypoxia tolerability through the HIF transcription factor, the nuclear translocation of which is facilitated by inactivation of tumor suppressors such as *VHL* and *p53* [14]. As mentioned above HIF induces transcription of several genes for glycan synthesis, leading to the significant alteration of glycan profiles, including enhanced sialyl $Lewis^{x/a}$ expression in cancer cells [128].

Another very interesting study has also implicated hypoxia as a determinant of glycogene expression in colon cancer. Koike and colleagues demonstrated that hypoxic culture of colon cancer cells induced a marked increase in expression of selectin ligands, the SLe^x and SLe^a determinants at the cell surface, which led to a definite increase in cancer cell adhesion to endothelial E-selectin. HIF was increased in colon cancer where it induced transcription of four important glycogenes — FucT-VII (*FUT7*), sialyltransferase ST3GAL1, and UDP-galactose transporter-1 (*UGT1*), which are all known to be involved in the synthesis of the carbohydrate ligands for E-selectin [128].

5.2. Hypermethylation

DNA methylation and histone deacetylation, the epigenetic mechanisms for suppression of normal gene transcription commonly observed in cancers, are proposed to underscore the aberrant expression of

glycosylation related genes seen in some cancers [126]. As previously mentioned, DNA hypomethylation leads to enhanced SLe^x production on MUC1 rendering colon cancer cells more favorable to liver metastasis. It has been shown that a change in cytosine methylation within the promoter of certain glycosylation related genes is responsible for the expression of cancer-associated carbohydrate antigens in gastrointestinal, colon, pancreatic, and breast cancer [142–144]. Further work is needed to advance our understanding of the link between the cancer glycome and epigenomics.

6. Clinical significance — diagnostics, prognostics and therapy

Given the large body of evidence that has accumulated to definitively implicate changes in glycosylation in the development and progression of certain cancers, there has been a focus on clinically applicable glycan targeting for diagnostic and prognostic purposes [145]. To date, this has taken the form of development of tumor-associated glycan markers as diagnostic and prognostic tools alongside a large focus on the development of vaccines in this area; however increasing attention is being focused on harnessing glycan specific changes in cancer to improve therapeutic strategies [20]. This has led to variable success due to the inherent challenges faced when studying protein glycosylation related to the complexity and diversity of glycan structures and also, in the past, due to lack of reliable high throughput tools for detailed glycan analysis and profiling. An in-depth review of the technologies that are available or becoming available to evaluate and develop these markers is beyond the scope of this review, however there are several useful reviews of this topic [12,20,145]. The mainstay of tools to study glycans remains the use of lectins that have an affinity for specific carbohydrate structures. Lectin based methods include immunohistochemistry, lectin blots, liquid chromatography and lectin microarrays. Despite much progress using lectins as analytical research tools, there has been an almost complete lack of clinically applicable high throughput tools to quantify serological glycan biomarkers.

Although several of the well known “tumor markers” used clinically are glycoproteins, it has only been in recent years that these have been analyzed for more specific glycoforms to increase the sensitivity and specificity of these tests. One such example is the identification of altered PSA glycosylation patterns in prostate cancer that can help to distinguish between significant and insignificant prostate tumors [146]. Carbohydrate determinates on glycoproteins and glycolipids have been shown in the past to serve as useful serum diagnostic and prognostic markers in a variety of cancers. SLe^a is important in adhesion of colon, rectal and pancreatic cancer cells to the endothelium while SLe^x was found to play a role in adhesion of lung, breast and ovarian cancer cells [127].

Recently, Becker and colleagues showed that the majority of primary patient AML blasts and leukemia stem cells express an E-selectin ligand [147]. E-selectin ligand expression appeared to be upregulated in relapsed as compared to newly diagnosed patients. A glycomimetic selective E-selectin inhibitor, GMI-1271, was able to overcome adhesion mediated chemotherapy resistance of AML *in-vitro* and reduce the leukemia burden of primary AML engrafted NODscid IL2Rgc −/− mice in combination with chemotherapy agents daunorubicin and cytarabine. They found that adhesion of primary AML blasts to E-selectin caused up-regulation of members of the Wnt and sonic hedgehog pathways, which could be inhibited by GMI-1271 [147]. Based on this data a phase I trial of GMI-1271 as a chemosensitizing adjunct to standard chemotherapy in AML is planned.

Other approaches to inhibiting E-selectin:selectin ligand interactions include the use of sialyltransferase inhibitors and aptamers [148]. Aptamers are oligonucleotide-based recognition molecules that have extraordinarily high sensitivity and selectivity towards their targets. First generation aptamers are currently in clinical trials as potential anti-cancer agents, anti-coagulants, anti-diabetic agents, and for treatment of macular degeneration, but so far only one aptamer (Macugen/pegaptanib) has been approved. Aptamers are selected using an *in-vitro* selection process, known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) using an initial, highly diverse library of oligonucleotide sequences that was simultaneously developed by Gold and Szostak [149,150]. It is possible that DNA-based aptamers against the sialic acid *N*-acetylneuraminic acid (Neu5Ac) may have potential as an E-selectin inhibitors and remodeling of the glycome in myeloid cells using inhibitors of sialyl- and fucosyltransferase has already shown promise as it results in loss of selectin binding and impaired leukocyte rolling [151]. Sialyltransferase inhibitors have also shown specific activity in cancer where they have been demonstrated to suppress tumor angiogenesis and cell metastasis in several models both *in-vitro* and *in-vivo* [152,83,153].

The use of single lectins for specific detection of glycans associated with certain malignancies has been helpful in some cases such as the application of concanavalin lectin (ConA) and Wheat germ agglutinin lectin (WGA) reactivity to p185 in breast cancer [154] or the measurement of T-antigen in cervical cancer using peanut agglutinin lectin (PNA) [155]. In the last decades the emergence of lectin arrays has made it possible to profile a glycoprotein and compare it with other samples in a high throughput manner. A variety of approaches have been used including application of lectins to an array for direct detection of glycans or lectin/antibody arrays where antibodies to potential glycoprotein markers are printed onto glass slides. These microarrays are hybridized against serum lectins to detect different glycan structural units on the captured glycoproteins in a sandwich assay format [156].

Mass spectrometry (MS) based methods remain the gold standard for identification and structural analysis of protein glycosylation. MS can also be used to quantify carbohydrates released from individual or multiple glycoproteins. This has been applied in breast cancer using a MALDI–MS based glycomic profile of permethylated glycans to detect biomarkers in patients' serum [1,157]. Focusing simply of glycosylation related genetic signatures in certain cancers has yielded interesting and potentially useful prognostic information. In multiple myeloma a distinct glycome of genes between normal and malignant plasma cells has been defined and this has been associated to distinct cytogenetic abnormalities in this disease [158].

As our understanding of the complex glycome of cancer cells increases glycome remodeling becomes an ever more attractive approach to manipulate the metastatic and immune evasion properties of these cells. Moving forward rapidly in this field requires additional advances in the reliable detection and quantification of glycan heterogeneity.

7. Summary

With recent advances in glyco-analytical technologies a greater understanding of the functional significance of seemingly minor changes in carbohydrate linkages on cell surface proteins and lipids has come to light. There has been renewed interest in glycosylation as a dynamic process that can evolve quickly and transiently to accommodate changes in the local microenvironment of the cell and facilitate adhesive and migratory interactions. Our understanding of changes in glycan determinants on cancer related proteins, such as mucins, selectin ligands and integrins, has uncovered a new layer of complexity, leading to a greater understanding of how this normal process is altered in cancer, and how these subtle alterations can have enormous implications for cancer cell metastasis, survival, proliferation and immune escape. This deeper understanding of the cancer glycome has led to the exploitation of glycosylation for therapeutic and prognostic applications in a wide array of solid and hematological malignancies and has the potential to greatly impact the field.

The current wave of novel emerging data in this field provides rationale for investigation of newly opened questions as well as revisiting of previously under-investigated topics using newly available tools. As mentioned, altered glycosylation of selectin ligands in cancer contributes to a metastatic phenotype, however much remains to be answered

about the functional interplay of glycosyltransferase dysregulation in the tumor microenvironment and research to date is just beginning to "scratch the surface". In-depth understanding of carbohydrate remodeling in cancer will require detailed profiling of glycosylation patterns in the context of the tumor microenvironment.

Research agenda

- ☐ The use of glycosylation inhibition in cancer, including inhibitors of sialyltransferases.
- ☐ The detection of specific glycoforms of adhesion molecules such as integrins in cancer that can serve as targets.

Conflict of interest

The authors declare no conflicts of interest for this work.

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