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Glycosylation of Cancer Stem Cells: Function in Stemness, Tumorigenesis, and Metastasis

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Abstract

Aberrant glycosylation plays a critical role in tumor aggressiveness, progression, and metastasis. Emerging evidence associates cancer initiation and metastasis to the enrichment of cancer stem cells (CSCs). Several universal markers have been identified for CSCs characterization; however, a specific marker has not yet been identified for different cancer types. Specific glycosylation variation plays a major role in the progression and metastasis of different cancers. Interestingly, many of the CSC markers are glycoproteins and undergo differential glycosylation. Given the importance of CSCs and altered glycosylation in tumorigenesis, the present review will discuss current knowledge of altered glycosylation of CSCs and its application in cancer research.

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Introduction

Every cell has a unique glycome signature dictated by specific timing, expression, and location of glycogenes, and is dependent upon the extent and availability of carbohydrates [1]. Glycosylation is one of the important co- and/or posttranslational modifications required for the normal biological functioning of cells [1]. Glycosylation takes place by the covalent modifications of proteins (glycoproteins) or lipids (glycolipids) with carbohydrates by the action of glycosyltransferases (GFs) and glycosidases in the endoplasmic reticulum (ER) and Golgi [1–3]. Further, glycoproteins and glycolipids (glycoconjugates) regulate a diverse range of biological and cellular activities, including pluripotency, embryogenesis, cell-to-cell and cell-to-environment interactions, signal transduction, protein folding, and immune modulation [4–7]. Alterations in glycosylation have been linked to tumor development and progression [5]. Aberrant glycome of tumors might also explain the heterogeneity seen in numerous cancers. Hakomori and Kannagi postulated that there are two main mechanisms for expression of tumor-associated carbohydrate antigens, specifically, incomplete synthesis (truncated glycans; Tn, sTn) and neosynthesis [*de novo* expression; sialyl Lewis a (sLe^a) and sialyl Lewis x (sLe^x)] [5, 8–10].

Recent studies support the involvement of CSCs in tumor development, metastasis, chemoresistance, and recurrence [11, 12]. CSCs or tumor-initiating cells are the rare, small subset of cells in the tumor with the ability to give rise to complete tumor masses [12]. CSCs can self-renew, can undergo asymmetric or symmetric cell division, and are associated with cellular heterogeneity [11–14]. They are thought to be derived from mutations in the stem or progenitor cell and hence tend to have the same stem cell markers [15], and various CSC markers are defined in many cancers to identify and isolate CSC populations [11]. Research has exploited membrane glycoproteins (CD44, CD24, ESA, CD133, etc.) to identify and sort CSC populations by using fluorescent antibody labeling and fluorescence activated cell sorting [16–18]. Another well-known method for isolating CSCs is Hoechst staining, the method by which cells are analyzed and sorted according to their ability to efflux the 33342 dye out of the cell. CSCs efflux the Hoechst dye due to higher levels of ABC transporters and appear as side populations (SP) in Hoechst red versus Hoechst blue plot in flow analysis [18, 19]. In recent years, researchers worldwide have accepted the existence of CSCs mainly because of tumor heterogeneity, chemoresistance, and tumor relapse. Present available drugs are efficient in only killing the bulk of tumor mass, sparing CSCs and leading to tumor recurrence and metastasis [12, 20]. There is thus an urgent need to develop new

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ways to characterize and understand the molecular mechanism of stemness of the CSCs in detail to target them. However, the role of glycosylation alterations in stemness and aggressiveness of CSCs has not been much explored.

In the present review, we discuss current knowledge of glycan modification of CSCs markers and its significance. We further present the significance of mucins in CSCs and finally discuss the few well-studied reports showing the role of GFs in regulating the self-renewal and stemness of CSCs.

Protein Glycosylation

Protein glycosylation is the attachment of carbohydrate to the amino acid (aa) residue of the protein backbone. There are many types of glycan modifications present in the cell, specifically, the *N*-, *O*-, *C*-linked, and *O*-GlcNAc modifications (Figure 1) [1]. The abundant and commonly occurring types of protein glycosylation include the *N*-linked, *O*-linked, and *O*-GlcNAclyation.

N-linked Glycosylation

In *N*-linked glycosylation, a glycan moiety (Glc2Man9GlcNAc2-) is added to the amino group of an asparagine residue (Axn-X-S/T, X= any amino acid except proline) of the polypeptide contained in the ER. The newly formed protein additionally undergoes proper folding followed by trimming, and the diversification of glycans takes place sequentially in the ER and Golgi, resulting in the synthesis of three main types of *N*-linked glycans: the high mannose, hybrid, and complex types (Figure 1) [2, 21].

O-linked Glycosylation (Mucin-Type *O*-Glycosylation)

Mucin-type *O*-linked glycosylation takes place by the addition of GalNAc to the hydroxyl group of serine or threonine (in the region of proline-rich) of the polypeptide in the cis-Golgi and subsequent addition of glycans in medial and trans-Golgi by the action of different GFs [1, 22]. This gives rise to the production of many core *O*-glycan structures, i.e., Core structures 1 through 8. Core 1-4 *O*-glycan structures are seen more abundantly in mammalian cells than are other core structures (Cores 5-8) [22] (Figure 1).

O-GlcNAcylation

In *O*-GlcNAcylation, GlcNAc is added to the serine or threonine residue of protein in the cytoplasm. Two enzymes regulate this type of glycan modification: O-GlcNActransferase adds the GlcNAc and *O*-GlcNAcase removes it. *O*-GlcNAcylation is commonly seen in the proteins that shuttle between the cell cytoplasm and nucleus (nucleocytoplasmic proteins). It has shown that phosphorylation and *O*-GlcNAcylation compete for the same serine or threonine residue in the polypeptide backbone of some proteins, such as P53, Myc, Pdx1, CREB1, and others, and that they regulate their differing functions (Figure 1) [4, 23].

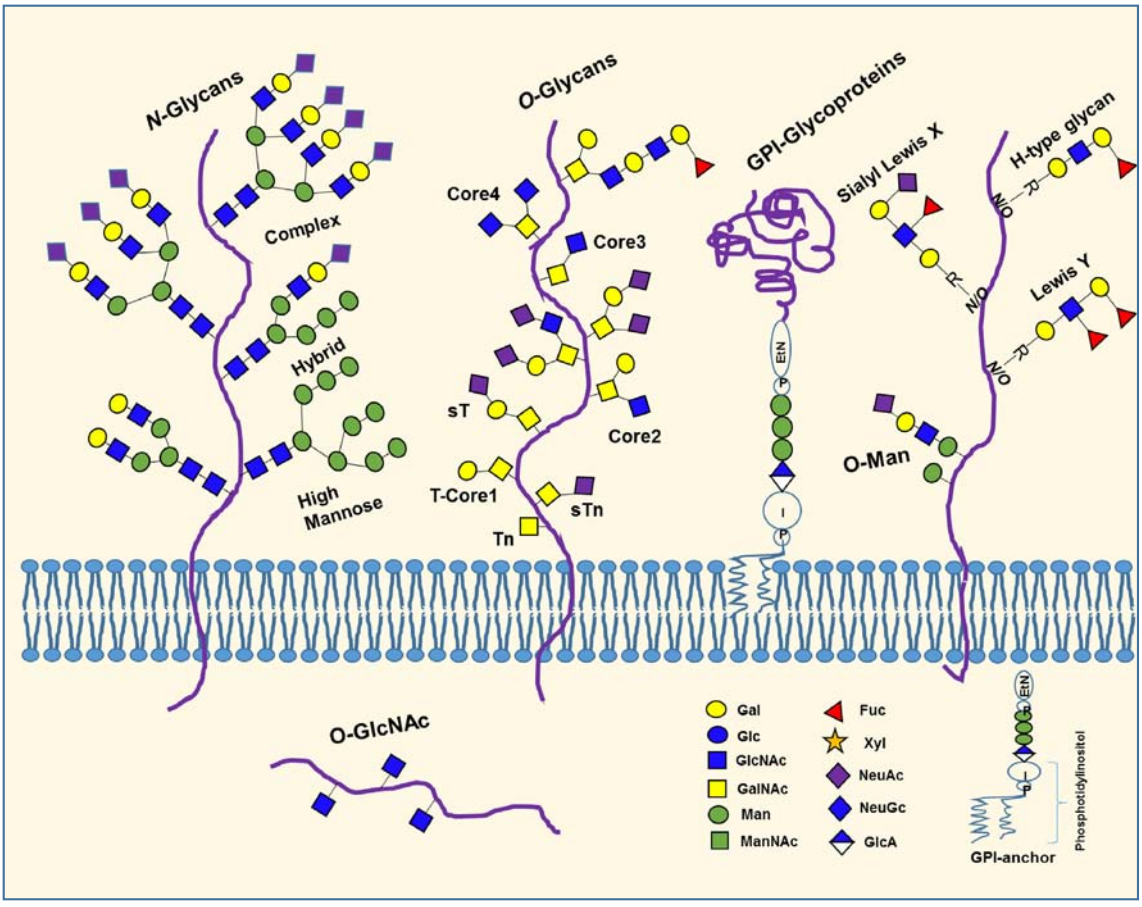

Figure 1. Glycan modifications on proteins. Depiction of different glycan modifications occurring in normal and cancer cells- N-linked glycoproteins (high mannose, Hybrid, complex type, β 1-6 branched), O-linked glycoproteins (Core-1, Core-2, O-Man, Tn, sTn, T, sT, sLeX), O-GlcNAc glycoproteins, glycosylphosphatidylinositol-anchored glycoproteins.

Pluripotency and reprogramming are regulated by *O*-GlcNAc modification of pluripotency-associated core proteins in embryonic stem cells (ESCs) [4].

Stem Cells and Glycosylation

Stem cells are undifferentiated cells that can convert into differentiated and specialized cell types. Two of the most critical features of the stem cells are pluripotency and self-renewal [24]. Stem cells are usually identified and sorted by the specific markers expression, and these markers may be cell surface or intracellular proteins, transcription factors, enzymes, etc. [25]. The role played by glycosylation in the embryonic development has been studied. Yan et al. have demonstrated *O*-fucosylation of Notch receptors to control blood lineage commitment [26]. In another study by Seth et al., they have demonstrated that core *O*-fucosylation of apolipoprotein B is required for proper midline patterning during zebrafish development by modulating the sonic hedgehog signaling [27]. These studies display the significance of glycosylation in mediating the embryonic development process.

ESC Markers and Its Glycosylation Variation

Many of the pluripotency-associated markers of ESCs are known to be glycoproteins or glycolipids, namely, TRA-1-60 and 1-81 and, stage-specific embryonic antigen 3 and 4. The glycans of these markers could be the potential modulator of pluripotency and stemness, which need to be explored in detail [28, 29]. A recent study by Jang H and colleagues has demonstrated the importance of glycosylation in regulating cellular pluripotency and reprogramming by modulating the core pluripotency-associated stem cell transcription factors in mouse ESCs [4]. This study also demonstrated that specific *O*-GlcNAc modification of pluripotency markers Oct4 and Sox2 occurs in the undifferentiated mouse ESCs and this glycan modification is lost upon differentiation. The *O*-GlcNAc modified Oct4 shown to enhance its transcriptional activity and regulate transcription of pluripotency-associated genes, resulting in maintenance of the pluripotent state of mouse ESCs and reprogramming of mouse embryonic fibroblasts [4]. The significance of glycosylation and glycan modification of stem cell transcription factor in regulating pluripotency in human ESCs and CSCs needs to be addressed.

Adult Stem Cell (ASC) Markers and Its Glycosylation Variation

Adult stem cells replace cells upon injury and maintain the tissue homeostasis, and it is not clear whether all the tissues of the body contain stem cells [30]. The role played by glycosylation in the maintenance of stemness in ASC has been studied. ESC marker Le^X (SSEA-1) is shown to express specifically on adult mouse neural stem cells (NSCs) [31]. Expression of Le^X antigen is identified on both glycolipid and glycoproteins and is shown to regulate the function of neural precursors cells [32]. Yagi and colleagues have demonstrated that *N*-glycans modified with Le^X to regulate mouse NSCs through modulating Notch signaling. Authors have shown that undifferentiated NSCs express the higher amount of Le^X carrying *N*-glycans compared to differentiated cells and are controlled by pax6 via upregulation of FUT-9 levels [33]. A detailed report on the role of glycosylation in stemness and differentiation of NSCs has been reviewed [34]. Hamouda et al. have characterized the *N*-glycans profile of undifferentiated and adipogenically differentiated in human bone marrow mesenchymal stem cells (MSCs). They have shown that *N*-glycans H6N5F1 and H7N6F1 are significantly higher expressed in undifferentiated than differentiated MSCs and identified as potential candidate markers [35]. In another study, CD44 modified SLe^X glycans on MSCs showed to facilitate their trafficking to bone [36]. A cell surface marker, CD133 is expressed and identified as a stem cell marker in hematopoietic stem cells (HSCs), progenitor cells, NSCs, and prostate stem cells [37–39]. The role of CD133 glycosylation is described in the glycosylation of CSC markers section. Another cell surface marker, CD44, is also identified as a stem cell marker of HSCs [40], and the importance of its glycosylation is described in the glycosylation of CSC markers section.

Glycosylation and Self-Renewal Pathways

Self-renewal is an essential phenomenon in which stem cells divide to give rise to more stem cells and maintain the undifferentiated state. Maintenance of self-renewal is attributed to the activation of many signaling pathways like leukemia inhibitory factor (LIF)/signal transducer and activator of transcription (STAT3), bone morphogenic protein 4 (BMP4)/Smad, Wnt/β-catenin and fibroblast growth factor 2 (FGF2), and activin/nodal in mouse ESCs and human ESCs [41]. Studies have shown the significance of glycosylation in the regulation of self-renewal pathways in ESCs. Sasaki et al. demonstrated that specific cell surface glycan LacdiNAc (GalNAcb1-4GlcNAc) contributes to self-renewal of mouse ESCs by regulating LIF/STAT3 signaling. Authors in this study showed that B4GalNAcT3 mediated LacdiNAc expression on LIFR and gp130 is required for induction and maintenance of self-renewal in undifferentiated mouse ESCs [42]. In another study, self-renewal and pluripotency of mouse ESCs are known to be regulated by cell surface proteoglycan heparin sulfate (HS). RNA interference-mediated knockdown of HS chain elongation resulted in the loss of self-renewal and differentiation of mouse ESCs. They also showed that HS regulates the expression of Nanog through auto/paracrine Wnt/β-catenin signaling [43]. Multiple studies have shown that HS is required for lineage commitment of ESCs and to modulate pluripotency [44]. Ligands involved in the activation of self-renewal pathways are known to be modified with glycosylation. Glycan modification of Wnt3a is required for its active form production and in turn activation of β-catenin–dependent Wnt signaling [45]. *N*-linked glycosylation of FGFR1 is shown to regulate its binding to ligand and co-receptor HS and, FGF signaling [46].

Glycosylation and Epithelial-Mesenchymal Transition (EMT) in Cancer

EMT is a remarkable phenomenon, which was initially observed to play a significant role in embryonic development and organ formation [47]. In the process of EMT, cells lose apical to basal polarity, change into fibroblastic nature, and display reduced epithelial markers and increased mesenchymal markers [47]. Several lines of research show the involvement of EMT in pathogenesis, particularly in tumor metastasis [48, 49]. The importance of glycosylation in regulating the EMT and cell migration process has been studied. Guan and colleagues have demonstrated decreased expression of GSLs, Gg4, and/GM2, and Gg4 synthase was observed in TGFβ-induced EMT process in mouse and human epithelial cells [50, 51]. Research by Freire-de-Lima and colleagues showed a direct correlation of *O*-glycosylation in regulating EMT process in human prostate epithelial cells. Authors have demonstrated TGFβ treatment to induce the expression of oncofetal fibronectin (onfN), GALNT-3, and GALNT6 activity and, in turn, *O*-glycosylation of onfFN resulting in the induction of EMT process [52]. A systemic

review by the same author has been published on the importance of aberrant glycosylation in cancer cells undergoing EMT process [53]. Huanna et al. have demonstrated that GALNT14 regulates the cellular proliferation, migration, and invasion by inducing the expression mesenchymal EMT genes and by stimulating MMP-2 activity in breast carcinoma [54]. In another study, *O*-GlcNAcylation of GNB2L1 protein is shown to regulate the metastasis via modulating the EMT proteins translation in the chemoresistance of gastric cancer [55]. Lucena et al. demonstrated a link between EMT and altered glycosylation through activation of hexosamine biosynthetic pathway. The authors have shown that cancer cells uptake more glucose during EMT through hexosamine biosynthetic pathway activation and in turn induce aberrant cell surface glycosylation (sialylationα2-6, poly-LacNAc, and fucosylation) and *O*-GlcNAcylation [56]. Role of specific *O*-glycan structures regulating the different function in tumor metastasis process has been reviewed. Tsuobai et al. have reported that core 2 O-glycans are helping in tumor metastasis by evading natural killer cells in circulation; in contrast, Core 3 O-glycans or O-mannosyl receptors suppress tumor metastasis by modulating integrin-mediated signaling [8]. Collectively, these studies display the significant role played by altered glycosylation in EMT and cellular migration process.

Glycosylation of CSC Markers

CSC markers are those molecules expressed at higher levels and used to identify and isolate CSCs from tumors [11]. CSC markers identified in numerous tumors are mainly cell surface glycoproteins, with the functional role of these glycan modifications being largely unknown [57].

CD44 and Its Glycosylation Variation

CD44 is a transmembrane glycoprotein that mediates lymphocyte homing and HA (hyaluronan)-dependent cell adhesion. The standard CD44 isoform (CD44s) is highly expressed in various cells types, including hematopoietic system. In contrast, the expression of variant CD44 isoforms (CD44v) is more limited. Both the standard and variant forms of CD44 actively contribute to the maintenance of stem cell populations by generating, embedding, and homing into a niche, establishing maintenance of quiescence and resistance to apoptosis [40]. Overexpression of CD44 in many tumors is implicated in tumor development [58], with CD44 identified as a universal CSC marker in many cancers, alone or with other markers such as CD24 and ESA [59].

The CD44 standard and its variants have been shown to be modified with *N*- and *O*-linked glycan modification. Moreover, the difference in the molecular weight of each isoform is linked to its differential glycosylation [60, 61]. Bartolazzi et al. demonstrated that five potential *N*-linked glycosylation sites on CD44 are required for CD44-mediated adhesion to HA in human cell lines [62]. Glycosylation of CD44 has been shown to regulate HA binding in ovarian tumors [63]. It has also been shown that glycosylation of CD44 has both stimulatory and inhibitory effects on cell surfaces and soluble CD44 binding to HA in the Chinese Hamster Ovary cell line ldl-D [64]. The *N*-linked *N*-acetylglucosamine residue, *O*-linked glycans (N-deglycosylated), and *N*-acetylgalactosamine incorporation into non–*N*-linked glycans on CD44 are importantly shown to amplify the binding of cell surface CD44 to HA. In contrast, α 2, 3-linked sialic acid on *N*-linked glycans inhibits CDD44 binding to HA [64]. Further studies showed that inhibition of *N*- and *O*-linked glycosylation of CD44 by tunicamycin (TM) and benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside reduces the attachment of endometrial cells to peritoneal mesothelial cells [65]. Expression of sLeX glycans on CD44 in MSCs also facilitates their trafficking to bone [36] (Figure 2*A*).

Expression of CD44 splice variants and their altered glycosylation are associated with metastatic properties of human tumors [58]. For instance, H-type glycan modification on CD44v6 produced by overexpression of the α1-2 fucosyltransferase gene resulted in increased tumor cell motility and tumorigencity in rat colon carcinoma cells [66, 67]. Modification of T and sTn antigens (*O*-linked glycosylation) was also seen in CD44v but not CD44s in colon cancer [68]. Further, modification of the T antigen on CD44 was also seen in higher amount in lung, breast, and liver cancer–initiating cells [69]. In breast cancer–initiating cells, co-expression of fucosylated Histo-Blood Group Antigens, CD173 (H2), and CD174 (Lewis Y), and CD44 has been reported [15, 70] (Figure 2*A*).

Importance of CD133 Glycosylation

CD133 (Prominin-1) is a cell surface marker that is expressed in HSCs and progenitor cell subpopulation but not in adult tissues [37]. Deregulated expression of this antigen was observed in several malignant hematopoietic diseases and in myelodysplastic syndrome. CD133 is widely used as a CSC marker in several malignancies [37]. Comparative genomics analysis on prominin-1 has shown that tandem TCF/LEF binding sites were conserved in PROM1 orthologs in human chimpanzee, mouse, and rat. The study proposes the involvement of CD133 in activation of WNT signaling in ESCs, adult, and CSCs [71]. Two monoclonal antibodies, AC133 and AC141, recognize glycosylated epitopes (undefined) of CD133 on the cell surface. These antibodies have been used to analyze and isolate CSC populations in many cancers, and a study demonstrated that, along with protein expression, the glycosylation status of CD133 may play a critical role in stem cell maintenance [72]. Further, studies showed that binding of AC133 is lost when CSCs differentiate and lose their stemness, but that this does not affect the change in mRNA and protein levels of human prominin-1 [73] (Figure 2*B*). In the same year as this study, Zhou and colleagues showed α2,3-sialylation to regulate the stability of stem cell marker CD133 in NSCs and glioma-initiating cells. They reported that CD133 was modified with *N*-linked glycans, with the terminal via α2,3-sialylation and desialylation with neuraminidases accelerating its degradation through the lysosome-dependent pathway [38]. AC133-negative glioblastoma cells have been shown to express a truncated prominin-1 variant protein, CD133, which is truncated with a molecular mass corresponding to ~16 kDa as detected by C24B9 (the anti-CD133 antibody) in the cytoplasm [74].

Another study demonstrated that *N*-linked glycan modification on CD133 regulated its cell surface localization and recognition by AC133. Differential glycosylation of *N*-glycan modification profiles was observed between CD133+ and CD133– cells. Enrichment of bi-antennary complex-type glycans and increase in the high-mannose type and terminal α2, 3-sialylation (ST3GAL6 overexpression) of *N*-glycans in CD133+ cells were observed [75]. Hypoxia was shown to induce the expression of CD133 by upregulation of OCT3/4 and SOX2 through HIF alpha signaling in human lung cancer cells [76]. In another study, hypoxia was shown to enhance glycosylation of CD133 in GSCs, and it was hypothesized that hyperglycosylated CD133 helped survival and invasiveness in GSCs [77] (Figure 3*A*). Liu et al. characterized the glycan sites of CD133 and demonstrated

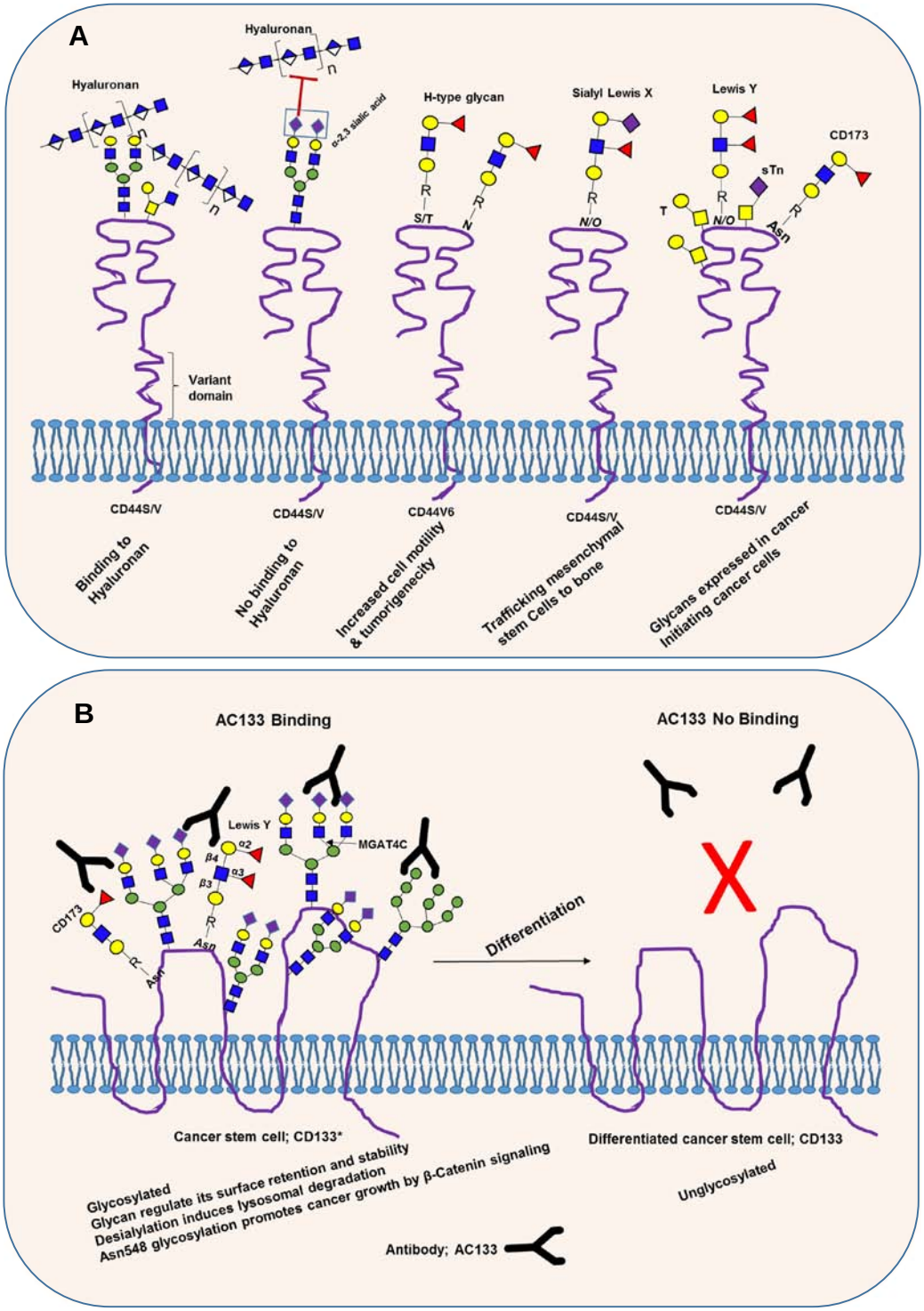

Figure 2. Glycan modification of CD44 and CD133. (A) Glycosylation of CD44 regulates its function in normal cells and cancer. *N*- and *O*-linked glycosylation of CD44 regulates HA binding. N-linked glycans with terminal α 2-3 sialic acid on CD44 inhibit binding to HA. H-type glycans on CD44v6 enhances cell motility and tumorigenicity. sLe^X modified CD44 mediates mesenchymal stem cells trafficking to bone. CD44 in cancer-initiating cells is shown to express truncate glycans like Tn, T, sT, and lewis Y and CD173. (B) CD133 glycosylation regulates its function in cancer and CSCs. *N*-linked glycans with α 2,3-sialic acids on CD133 regulate its cell surface retention and stability, and desialylation induces its lysosomal degradation. N-linked glycosylation at Asn548 enhances tumor growth through β-catenin signaling.

that loss of *N*-glycosylation at Asn548 reduced prominin-1, promoted cell growth and its association with β-catenin, and in turn inhibited β-catenin signaling in liver cancer. *N*-liked glycosylation sites of CD133 identified by mass spectrometry analysis were Asn206, Asn220, Asn274, Asn395, Asn414, Asn548, Asn580, Asn729, and Asn730 [78]. Co-expression of T antigen and CD133

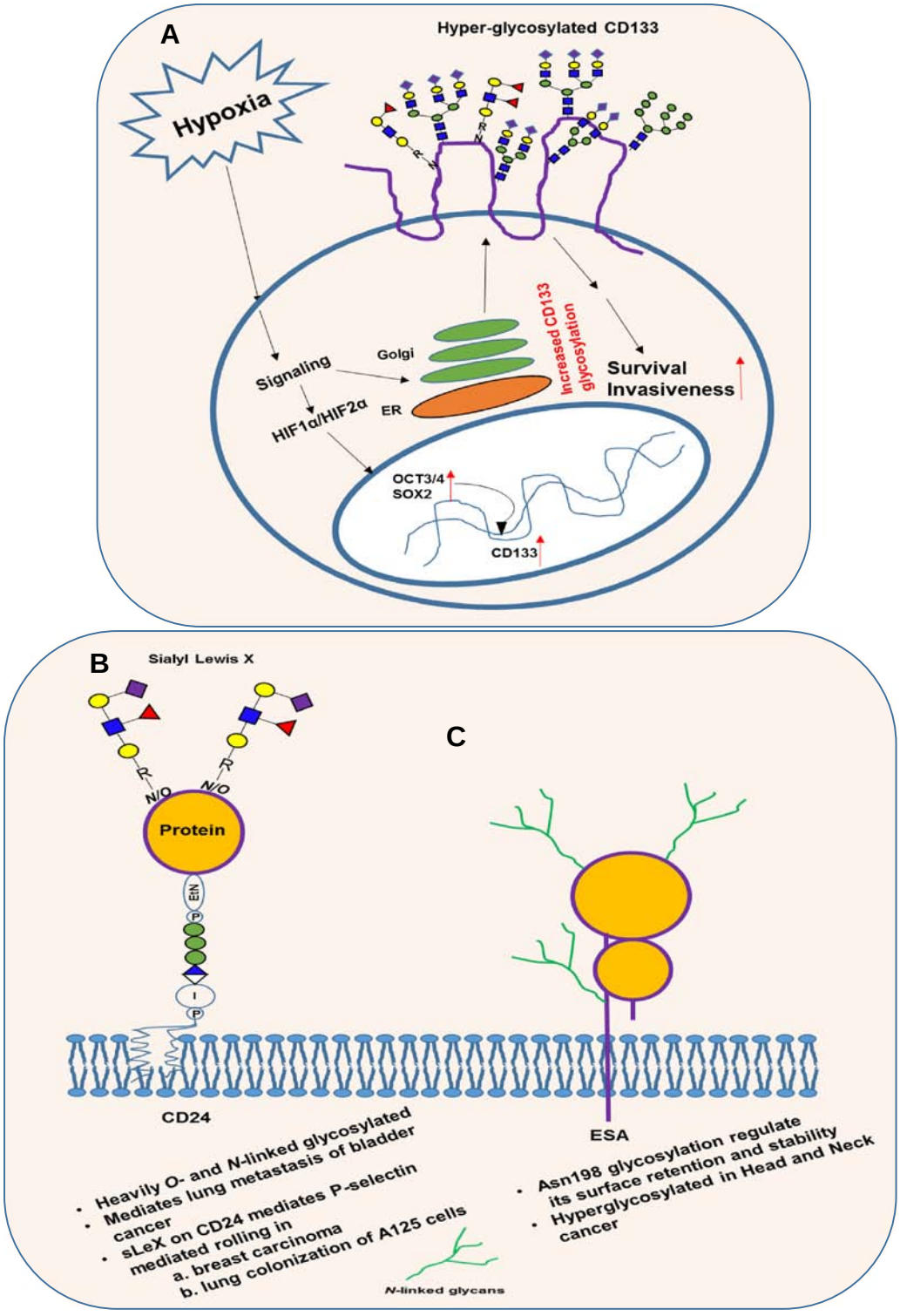

Figure 3. Glycan modification of CD133, CD24, and ESA. (A) Hypoxia enhances survival and invasiveness by inducing expression and hyperglycosylation of CD133 through Hif1α/Hif2α/OCT3/4/SOX2 signaling axis. (B) Glycosylation of CD24 mediates metastasis. CD24 is modified with *N*- and *O*-linked glycans. sLe^X modifies CD24-mediated, P-selectin-mediated rolling and lung colonization. (C) Glycosylation of ESA regulates its function in cancer. *N*-linked glycosylation at Asn198 on ESA regulates its surface retention and stability. ESA is hyperglycosylated in cancer compared to normal tissue.

was seen in lung, breast, and liver cancer–initiating cells [69]. Co-expression of CD173 (H2) and CD174 (Lewis Y) with CD133 has also been reported in breast cancer–initiating cells [70].

Surface Marker CD24 and Glycosylation Variation

In mice, CD24 was discovered as a heat-stable antigen and identified as a marker to differentiate hematopoietic cells and neuronal cells [79, 80].

CD24 (B cell differentiation marker) is a cell surface glycoprotein linked to glycosylphosphatidylinositol, mainly expressed on human B cells and in many tumors. It consists of a small protein core of 27 aa's that are heavily glycosylated with *N*- and *O*-linked type of glycans [81, 82]. Expression of CD24 was observed in higher levels in various human cancers and was involved in the cell adhesion, tumor progression, and metastasis [83–86]. CD24 was identified as a ligand for an adhesion receptor, P-selectin, on platelets and endothelial cells [87], through which it helps in extravasation of tumor cells in circulation. Further, CD24 increases tumor cell proliferation, and it shows increased adhesion to fibronectin, collagen, and lamin [84]. Cells expressing CD24+ are identified as CSCs in ovarian and colorectal cancers [88]. Myriad studies have shown vital role of CD24 in ovarian cancer metastasis, establishing it as a potential new CSC marker [89–92]. Gao et al. demonstrated that 5000 CD24+ cells form tumors in animal models with higher expression of stemness genes and found no tumorigenicity with the same number of CD24– cells [91]. Similarly, 500 CD24+ cells were shown to form tumors in mice models and express stemness genes, and were identified as CSCs in human nasopharyngeal carcinoma [93]. In another study, human ovarian cancer cell lines with phenotypes of CD44+CD24+EpCAM+ showed enrichment for stem/progenitor cells clonogenic capacity. A total of 0.5% to 1% of CD44+CD24+EpCAM+ cells were identified as CSCs in pancreatic cancer cells [94]. In contrast, CD44-high and CD24-low cells were identified as CSCs in breast and prostate cancer [95, 96]. The importance of CD24 glycosylation in regulating its function in cancer has been studied. CD24 modified with sLe^x was shown to meditate P-selectin–dependent rolling in breast carcinoma *in vitro* and *in vivo* [87]. CD24 with sLe^x modification also mediates P-selection–dependent rolling and lung colonization of human A125 adenocarcinoma cells [97]. CD24 further mediates the development of lung metastasis of bladder cancer [98] (Figure 3*B*), further showing the involvement of glycans on CD24 to mediate tumorigenesis and metastasis.

Role of Epithelial Cell Adhesion Molecule (EpCAM) in CSCs Maintenance and Glycosylation Variation

EpCAM or epithelial surface antigen (ESA) is a cell surface glycoprotein overexpressed in multiple tumors and in CSCs [99]. EpCAM promotes cell cycle and proliferation by upregulating the proto-oncogene c-myc and cyclin A or E [100]. EpCAM also regulates cellular metabolism by upregulating the fatty acid-binding protein E-FABP and contributes to carcinogenesis [101]. EpCAM is involved in the maintenance of hESCs in the undifferentiated phenotype by directly regulating few reprogramming genes, including c-MYC, OCT-4, NANOG, SOX2, and KLF4 [102]. In contrast, one study identified EpCAM only as a surface marker to identify undifferentiated hESCs as silencing of this gene did not affect the levels of pluripotent marker [103]. EpCAM was shown to be *N*-glycosylated at the three-glycosylation sites: Asn74, Asn111, and Asn198 in human epithelial cells. In another study, EpCAM was shown to be *N*-glycosylated at Asn88 and Asn51 expressed in insect cells [99, 104]. In head and neck cancer, EpCAM has been reported to be hyperglycosylated with *N*-linked glycans compared to autologous normal epithelia [105]. EpCAM hyperglycosylation at Asn198 regulates its protein stability and cell surface retention in HEK293 cells [106]. Furthermore, *N*-glycosylation of EpCAM has been shown to regulate apoptosis in breast cancer cells, as de-glycosylation of EpCAM promoted apoptosis and inhibited cell proliferation of breast cancer cells [107] (Figure 3*C*).

CSCs and Mucins

Mucins are heavily glycosylated proteins carrying greatly *O*-linked glycans with few *N*-linked. *O*-linked glycosylation mainly takes place on serine, threonine, and proline-rich regions of variable tandem repeat regions of mucins. Mucin expression is commonly seen on epithelial cells, where they have primary protective functions against microbial infections [5, 108]. Deregulated mucin expression has been linked to the pathogenesis of many diseases, including cancer. Mucins such as MUC1, MUC4, MUC5AC, and MUC16 are some of the well-studied *O*-linked glycoproteins for tumor-promoting potential [109–113]. Aberrant glycosylation of mucins has been associated with cancer development and progression [5, 9, 114]. The role of mucins and their altered glycosylation in CSCs has not been explored.

MUC1, a transmembrane glycoprotein, is overexpressed and aberrantly glycosylated in many cancers. MUC1 contains mainly core 2, its elongated glycan structures in normal cells; however, expression of truncated and neo-glycan structures is observed in cancer. This aberrant glycosylation of MUC1 activates oncogenic signaling in cancer [115]. N-terminal cleaved mucin 1 (MUC1) is expressed only in undifferentiated human pluripotent stem cells and mediates its growth by acting as a growth factor receptor [116]. MUC1 expression has been observed in the CD44+CD24+ESA+ and CD133+ CSCs of pancreatic cancer [117]. Overexpression of MUC1 has been reported in human stem cells fraction of cord blood cells and in many acute myeloid leukemia (AML) cases. MUC1 has been shown to increase frequencies of progenitor and long-term culture-initiating cells [118]. MUC1 overexpression is seen only in AML stem cells and not in normal stem cell counterparts; targeting of MUC1C by GO-203 has been shown to deplete AML *in vivo* [119]. Expression of the hypoglycosylated form of MUC1 expression was reported in the SP of MCF7 breast cancer cells [120]. MUC1 overexpression and $CD44^+/CD24^-$ cancer stem-like cell enrichment were observed in response to exposure of tumor-associated macrophages to breast cancer MCF7 cells [121]. Apoptosis of MCF7 cells triggered by staurosporine has been shown to activate $CD44^+/CD24^-$ cancer stem-like cells by increasing expression of ESA and MUC1 [122] (Figure 4*A*).

Mucin 4 is also aberrantly expressed in many cancers and has been identified as a diagnostic cancer marker [114, 123]. MUC4 maintains CSC population in ovarian cancer by stabilizing Her2 expression. Its overexpression increases the SP and CD133+ CSCs of ovarian cancer [124]. MUC4 overexpression also increases the CD133+ CSCs population of pancreatic cancer and was shown to provide gemcitabine resistance [125] (Figure 4*B*).

Another transmembrane glycoprotein, MUC16 (CA125), a heavily glycosylated and large mucin, is implicated as having a tumor-promoting role in many cancers, including ovarian and pancreatic [110]. MUC16-expressing cells are identified as the source of CSCs in ovarian cancer. Studies showed that only $CA125^+/lineage^-$ cells form tumors but not $CA125^-/lineage^-$ cells in mouse orthotopic implantation [126]. In another study, the role of MUC16 in the enrichment of CSC populations and in tumorigenesis, and its metastatic potential in pancreatic cancer were demonstrated [110]. MUC16-cter mediates upregulation of stemness genes such as NANOG and LMO2 through JAK2 nuclear translocation and histone 3 phosphorylation, and it maintains stemness [127] (Figure 4*C*).

GFs in Stemness of CSCs

Expression of a specific set of glycogenes at a time in a particular cell type determines the resultant signature of glycome on protein or lipid

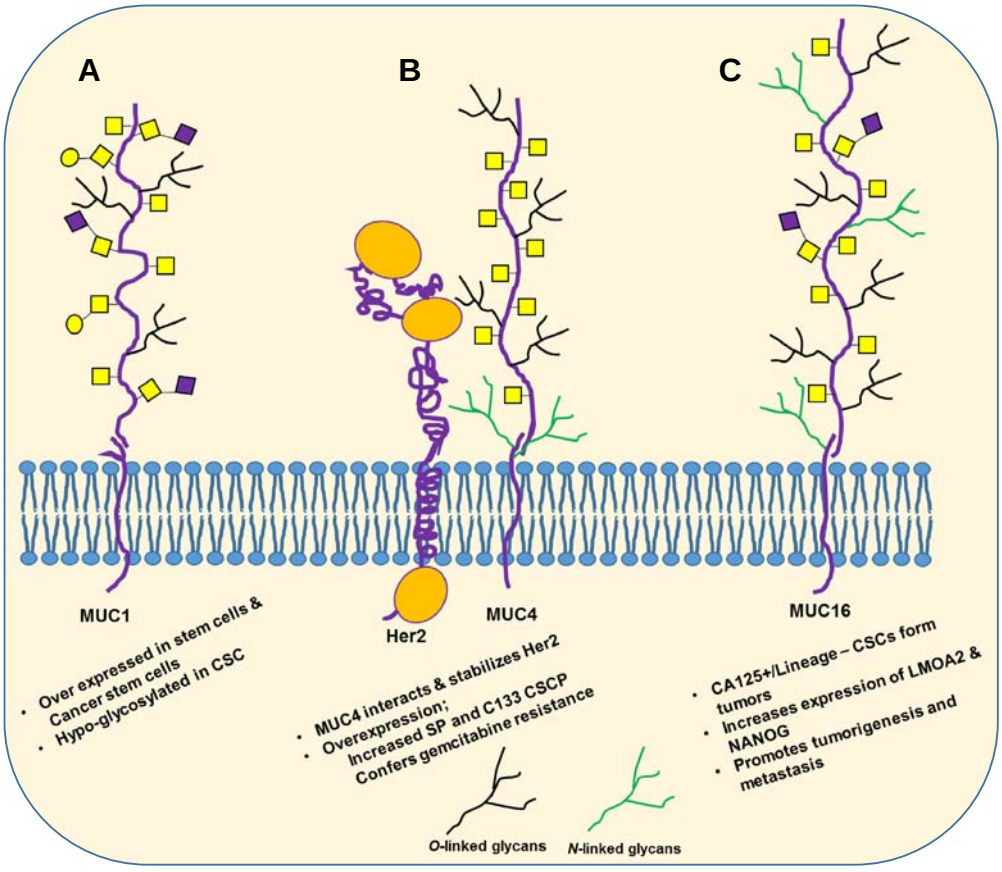

Figure 4. Role of mucins in CSCs. (A) Mucin 1 is overexpressed in human pluripotent stem cells and CSCs. Mucin 1 is hypoglycosylated in a CSC population. (B) Mucin 4 expression increases CSC population and provides drug resistance by Her2-mediated signaling. (C) Mucin 16 expressing cells identified as CSCs and Mucin 16 expression shown to regulate stem cell transcription factors LMOA2 and NANOG through the JAK/STAT pathway.

molecules. The different glycans on the cell membrane drive the diverse cellular signaling. Expression of glycogenes, specifically GFs, has been linked to the development and progression of many cancers [9].

One well-studied GF for the stemness of CSCs is glucosylceramide synthase (GCS). GCS is highly upregulated in breast, colon, leukemia, and various drug-resistant cancer cells. GCS catalyzes ceramide glycosylation, the rate-limiting step in glycosphingolipid synthesis. Reports have shown that inhibition of GCS sensitizes cancer cells to anticancer drugs and eliminates CSC population by modulating gene expression, reducing MDR1, and restoring expression of p53 via RNA splicing [128]. In breast CSCs, increased expression of ceramide glycosylation and globotriosylceramide (Gb3) was observed with the overexpression of GCS. This higher level of Gb3 was also shown to upregulate FGF-2, CD44 expression, and Oct4 and to maintain stemness of breast CSCs through c-Src/β-catenin signaling. Silencing of GCS was also shown to disrupt Gb3 and kill breast CSCs [129] (Figure 5*A*). Liu YY and colleagues reported that inhibition of GCS led to increased ceramide levels in cells and restoration of the expression of wild-type p53 resulting in activation of p53-dependent apoptosis [130, 131]. Liu's group has also shown that inhibition of GCS led to the expression of wild-type p53 and that it abolished the p53 R273H mutant-derived EMT and induced pluripotency of colon cancer [132] (Figure 5*A*).

β1,4-N-acetylgalactosaminyltransferase III (B4GALNT3) is overexpressed in the colon CSCs, which is involved in the synthesis of LacdiNAc structures. A study indicated that LacdiNAc structures play a role in the self-renewal of mouse ESCs [42]. B4GALNT3 was reported to modify N-glycans of EGFR with LacdiNAc and regulates stemness, migration, and invasiveness of CSCs. The knockdown of B4GALNT3 also decreased expression of the stem cell markers OCT4 and NANOG in colon cancer cells [133] (Figure 5*C*). Another GF, MGAT5 (GnT-V), is also shown to promote tumor development in many cancers, including colon carcinoma [9]. MGAT5 synthesizes *N*-glycans with β-(1,6)-branching and is involved in the development of many tumors by the way in which it modulates the function of various cell surface receptors and their intracellular signaling pathways [9]. In colon CSCs, MGAT5 was shown to modify the Wnt receptor, FZD-7, with β-(1,6)-branched N-glycans, thus affecting Wnt signaling, CSC compartments, and tumor progression [134]. Reduced colon (intestine) CSC populations in NOD/SCID mice were also interrelated with lower levels of MGAT5. Significantly reduced adenoma size and survival of MAGT5 knockout APCmin/+ mice were also observed in the study [134] (Figure 5*C*). Pancreatic CSCs were reported to overexpress the enzymes involved in the synthesis of fucosylated glycans such as fucosyltransferases (Fut1-4), GDP-fucose synthetic enzymes (FX, GMDS), and GDP-Fucose transporters [135]. In this study, authors reported an increase in the expression of α1,2- and α1,3-/α1,4-

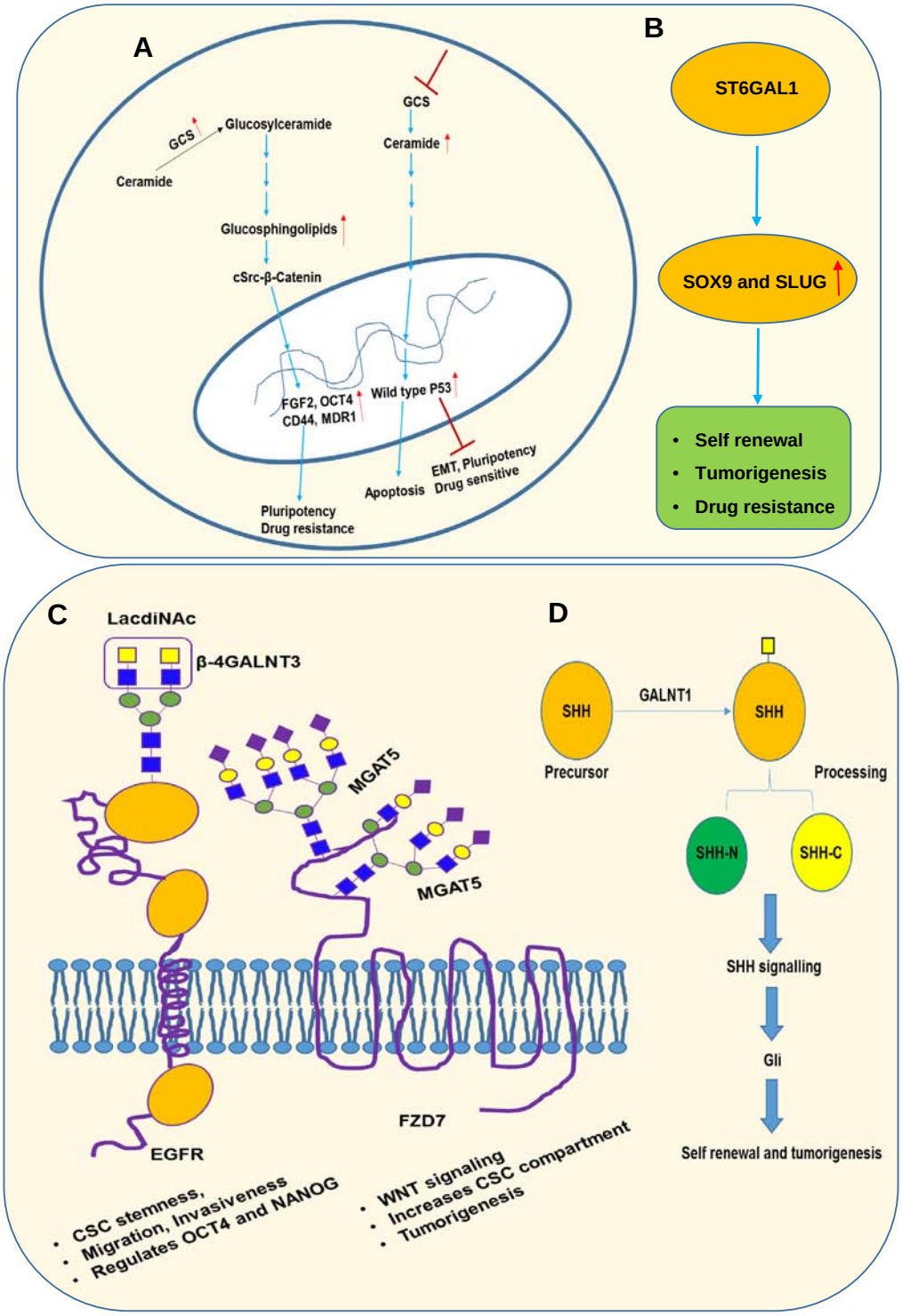

Figure 5. GFs regulating stemness in CSCs. (A) Glucosyl ceramide synthase regulates pluripotency and drug resistance through cSrc-β-catenin signaling. (B) ST6GAL-1 regulates stemness and drug resistance by inducing expression of SOX9 and SLUG. (C) B4GALT3 regulates stemness by inducing expression of OCT4 and NANOG by modifying EGFR with LacdiNAc. MGAT5 regulates stemness and tumorigenesis by modifying WNT receptor with β-1-6 branched N-linked glycans. (D) GALNT1-mediated modification of GalNAc on SHH induces expression of gli1 and regulates stemness and tumorigenesis.

fucosylated glycans by lectin array [135]. In pancreatic and ovarian cancer, ST6Gal-1, which adds α 2-6 sialic acid on substrate glycoproteins, is shown to confer CSC phenotypes by regulating the stem cell transcription factors Sox9 and Slug and to augment tumor-initiating potential and resistance to gemcitabine [136] (Figure 5*B*). In bladder CSCs, GALNT1 has been shown to regulate the self-renewal and maintenance of bladder CSCs (BCMab1+CD44+) and bladder tumorigenesis by modifying *O*-linked glycosylation and activating SHH signaling through Gli1. Inhibition of bladder tumor growth was also observed by the intravesical instillation of GALNT1 siRNA and cyclopamine, an SHH inhibitor [137] (Figure 5*D*).

Conclusion and Future Prospective

In the present review, we have provided current knowledge of the glycosylation of CSCs in the maintenance of stemness, tumor development, and metastasis. In CSCs, glycan modification of specific markers plays several roles, including adhesion, survival, invasiveness, metastasis, pluripotency, stemness, drug resistance, and apoptosis. Mucin glycoproteins and GFs also regulate stemness, tumorigenesis, drug resistance, and metastasis in CSCs. However, the glycan modification on these mucins by specific GFs and its involvement in CSCs maintenance are unclear and require exploration.

Glycome, the total glycan signature of a cell, is unique for each cell type. Glycans play a critical role in regulating diverse cellular functions, and it is therefore prudent to study the glycome of normal stem cells, CSCs, and non-CSCs so as to exploit the differences in glycomes. The aberrant glycosylation can be used as a biomarker for early detection or to specifically target CSC populations. Understanding the role of CSC-specific GFs, the tumor-associated carbohydrate antigens on CSC markers, and glycoproteins, including mucins, will further open opportunities to identify new targets and strategies for early detection and targeted therapeutics. Altered glycans on CSCs, for example, can be transferred clinically to a glycopeptide-based vaccine. These vaccines, in combination with FDA-approved cytotoxic drugs, will help to eradicate CSCs and cancer cells in present cancers and, critically, to inhibit tumor recurrence in many susceptible cancers.

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Ethical Approval

This review article does not contain any studies with human participants and animals.

Conflicts of Interests

The authors declare no conflicts of interests.

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Author Contributions

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