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Glycosaminoglycans: key players in cancer cell biology and treatment

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This article is dedicated to Professor Kazuyuki Sugahara (Laboratory of Proteoglycan Signaling and Therapeutics, Faculty of Advanced Life Sciences, Hokkaido University, Sapporo, Japan) for his significant contribution and research achievements in the fields of structural analysis and biological roles of glycosaminoglycans.

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Glycosaminoglycans are natural heteropolysaccharides that are present in every mammalian tissue. They are composed of repeating disaccharide units that consist of either sulfated or non-sulfated monosaccharides. Their molecular size and the sulfation type vary depending on the tissue, and their state either as part of proteoglycan or as free chains. In this regard, glycosaminoglycans play important roles in physiological and pathological conditions. During recent years, cell biology studies have revealed that glycosaminoglycans are among the key macromolecules that affect cell properties and functions, acting directly on cell receptors or via interactions with growth factors. The accumulated knowledge regarding the altered structure of glycosaminoglycans in several diseases indicates their importance as biomarkers for disease diagnosis and progression, as well as pharmacological targets. This review summarizes how the fine structural characteristics of glycosaminoglycans, and enzymes involved in their biosynthesis and degradation, are involved in cell signaling, cell function and cancer progression. Prospects for glycosaminoglycan-based therapeutic targeting in cancer are also discussed.

Abbreviations
CS, chondroitin sulfate; CS-A, GlcA-GalNAc-4-sulfate; CS-B, dermatan sulfate; CS-C, GlcA-GalNAc-6-*O*-sulfate; CS-D, [GlcA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate)]; CS-E, GlcA-GalNAc-(4,6)-*O*-disulfate; CSPGs, chondroitin sulfate proteoglycans; DS, dermatan sulfate; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAGs, glycosaminoglycans; GlcA, glucuronic acid; GlcN, glucosamine; HA, hyaluronan; HAS, hyaluronan synthase; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; HSulf, human sulfatase; Hyal, hyaluronidase; IdoA, iduronic acid; MMP, matrix metalloproteinase; MT3-MMP, membrane type 3 matrix metalloproteinase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PG, proteoglycan; PI3K, phosphoinositide 3-kinase; TGF, transforming growth factor; TNF, tumor necrosis factor; UDP-GlcA, UDP-D-glucuronic acid; VEGF, vascular endothelial growth factor.

Introduction

Cancer is recognized as one of the leading causes of mortality worldwide. It is well established that interaction among the various molecules expressed by cancer cells is influenced by effectors/stimuli that are secreted by stromal cells within the tumor microenvironment [1,2]. These interactions may affect tumor growth and cancer progression (invasion and metastasis). The signals transmitted to the cell closely affect the gene expression profile of macromolecules as well as cell function.

The extracellular matrix (ECM) not only provides tissue support, its functional macromolecules are also involved in the regulation of cell properties and function. This is mainly due to interactions between ECM components and cell-surface receptors, growth factors and cytokines. Matrix cell-surface molecules, such as syndecans and integrins, may also themselves act as cell receptors/co-receptors. Therefore, ECM constituents are closely involved in the cellular and molecular mechanisms of cancer cells, and affect their adhesion and migration as well as their invasiveness and metastatic potential.

Proteoglycans (PGs) are present in almost all tissues at the extracellular and cellular (cell membrane and intracellular) levels. PGs are one of the best-studied classes of matrix molecules, and more than 50 members have been characterized and studied for their biological significance. They comprise a protein core to which glycosaminoglycan (GAG) chains are covalently linked. GAGs are linear, negatively charged heteropolysaccharides composed of repeating disaccharide units of *N*-acetylated hexosamine and uronic acid. Differences in the type of monosaccharide in the repeating unit as well as their sulfation result in the following major categories of GAGs: hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS), heparin and heparan sulfate (HS) and keratan sulfate. Keratan sulfate lacks uronic acid and contains galactose in its disaccharide building blocks. GAG chains are covalently bound to serine residues of the PG protein core via a tetrasaccharide linkage, consisting of xylose, two galactose residues and glucuronic acid. In contrast with other GAGs, HA, a non-sulfated glycosaminoglycan, lacks a covalently bound protein core, and is synthesized at the intracellular surface of the plasma membrane. This macromolecule is considered to be a key player in tissue homeostasis and also in cancer progression and inflammatory conditions [3]. Sulfated GAGs are synthesized at the Golgi apparatus and modified by *O*-sulfotransferases. The inverse levels of two main biosynthetic transferases, exostoses (multiple)-like 1 (EXTL1) and chondroitin sulfate N-acetylgalactosaminyltransferase 1 (CSGalNAcT-1), involved in the synthesis of HS and CS, respectively, observed during B-cell differentiation, suggest a developmentally regulated switch between CS and HS synthesis [4]. Various sulfation patterns have been documented at various sites of the hexosamine and uronic acid GAG components, promoting enormous structural diversity and complexity [5,6].

We describe below the role and function of GAGs in cancer progression and cell signaling. The importance of GAGs in therapeutic strategies, together with future perspectives, are also discussed.

HS in cancer progression and cell signaling

HS proteoglycans: structure and function

Heparan sulfate is present in all cell types and tissues and acts as a regulatory molecule in numerous normal and pathological conditions. HS consists of repeating disaccharide units of glucosamine (GlcN) and hexuronic acid residues [glucuronic acid (GlcA) and iduronic acid (IdoA)] (Fig. 1). Hydroxyl groups, as well as the amino group of glucosamine, are all available positions for sulfonylation. Twelve sulfation profiles for HS/heparin disaccharides are known and have been resolved and characterized by various separation techniques [7–9]. Sulfation motifs of HS chains are of crucial importance for the interactions of HS with growth factors, cytokines and proteins, and therefore the sulfation pattern of domains within HS/heparin chains may affect their biological roles [9]. HS chains are covalently bound to a protein core to form HS proteoglycans (HSPGs) [10].

Two main categories of HSPGs have been recognized according to their cell localization; cell-surface PGs (syndecans and glypicans) and basement membrane PGs (perlecan, agrin and collagen type XVIII). HSPGs modulate multiple functions of tumor cells by promotion of important transformations in cell phenotype, leading to tumor growth, invasion and metastasis [2,10,11]. They may interact with several bioactive molecules, including growth factors, chemokines, cytokines, morphogens and enzymes, through their HS moieties [12]. The ability of various ligands to bind HSPGs depends to a large extent on the fine structure of their HS chains. The disaccharides are distinguished by the presence of sulfated or non-sulfated GlcA/IdoA and GlcN residues.

The multiple roles of HSPGs, which have been shown to be related to the HS chains, involve organization of the ECM through binding with other matrix molecules (e.g. collagen I and IV, fibronectin and laminin), as well as the structural development and consistency of the ECM and basement membrane. It is worth noting that HS is a modulator for cell–cell and cell–ECM interactions. It has been shown that the presence of HS chains promotes cell adhesion, whereas their absence promotes invasion and migration of malignant cells [13,14]. HS can also mediate the bioavailability of selected growth factors and peptides through depository inactivation [15] or by gradient diffusion of them in cell-surface receptors. Growth factors interact with HSPGs, creating a protected 'reservoir' of growth factors that are released in certain cases, promoting or suppressing tumor progression and angiogenesis.

The importance of HS structure in the biological roles of HSPGs and growth factor-mediated signaling

The complexity of HS moieties and the HS-mediated interactions have been well-illustrated for fibroblast growth factor–fibroblast growth factor receptor (FGF2–FGFR) signaling. The binding of FGF2 and FGFR to HS chains occurs through *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid units [16], or 6-*O*-sulfated glucosamine [17]. Many studies have highlighted modifications in HS sulfation during cancer progression. For example, in melanoma cells, FGF2 stimulated cell proliferation, triggered by the highly sulfated HS [18]. Moreover, crystal structure analysis has revealed binding of FGF2 to tetra- or hexasaccharide motifs of HS and even longer oligosaccharide sequences that act as a bridge between ligands and receptors [17]. On the other hand, binding of HS to hepatocyte growth factor, platelet-derived growth factor, lipoprotein lipase and herpes simplex virus glycoprotein C was dependent on 6-*O*-sulfation [18]. The activation of antithrombin III by HS/heparin is mediated by a specific pentasaccharide in which a 3-*O*-sulfate group is crucial [19–21].

The presence of HS chains in cell-surface PGs is of particular significance, as they regulate cancer progression. Cell-surface HSPGs consist of two sub-classes, syndecans and glypicans, that are transmembrane and glycosylphosphatidylinositol-anchored PGs, respectively. Syndecan-1 and syndecan-4 have been implicated in the promotion of signaling through formation of complex HS moieties with FGF2 and FGFR-1 in tumor progression [22]. It has been established that syndecan-4 expression modulates focal adhesion kinase (FAK) Y397 phosphorylation, and promotes melanoma cell adhesion and migration through its HS chains [23].

With regard to glycosylphosphatidylinositol-anchored glypicans, it has been shown that over-expression of glypican-3 in hepatocellular carcinoma and melanoma induces tumor growth signaling upon binding of its HS chains to Hedgehog protein and Wnt. In contrast, glypican-3 in its shed form is able to block Wnt signaling and subsequently inhibit hepatocellular carcinoma. It is worth noting that interactions between the HS chains of glypican and heparin-related growth factors attenuate tumor growth and invasion, and, moreover, that the HS chains are essential for glypican-induced stimulation of FGF [24,25].

Changes in the expression pattern of matrix macromolecules provide the cancer cells with the ability to invade and metastasize, but, prior to these events, the morphology of the carcinoma cells changes from epithelial to mesenchymal [26]. The HS chains play a key role in the epithelial to mesenchymal transition due to their high ability to bind key growth factors secreted into the tumor microenvironment by primary tumor cells, fibroblasts (FGF, hepatocyte growth factor and insulin growth factor), macrophages (epidermal growth factor, EGF), inflammatory cells (tumor necrosis factor α, TNFα), myofibroblasts and epithelial cells (transforming growth factor β, TGFβ) [27,28]. This has been well demonstrated in the case of hepatocyte growth factor, which forms a triple complex with HS chains and its high-affinity receptor c-MET, affecting the signaling cascade associated with tumor invasion and metastasis [29].

The structural alterations of GAGs are highly coordinated with the action of specific enzymes responsible for the metabolism of HS chains. The over-expression of degrading enzymes can lead to mechanisms that promote invasion and metastasis [30,31]. Heparanase, an endo-β-glycosidase that cleaves HS side-chains of HSPGs is one such enzyme. This molecule arranges ECM disassembly and mediates binding to growth factors involved in several fundamental functions. In this way, it induces signaling pathways correlated with cell proliferation, metastasis and angiogenesis, as well as inflammation [31,32]. Research data have highlighted the synergistic role of heparanase and vascular endothelial growth factor (VEGF) in promoting melanoma progression via the MEK/extracellular signal-regulated kinase (ERK) signaling pathway [33]. Heparanase also appears to regulate the expression of VEGF in several cancer cell types via the Src kinase family [34]. In the case of head and neck carcinomas, heparanase enables the phosphorylation of Src and epidermal growth factor receptor (EGFR) through its C-terminal domain

regardless of its enzymatic activity, leading to activation of the intracellular transcription factors STAT3 and STAT5b, but not STAT5a [35]. Moreover, differential regulation of Ras-related C3 botulinum toxin substrate 1/Ras homolog gene family member A (Rac1/RhoA) activities by heparanase affects medulloblastoma cell proliferation and invasion [36]. Heparanase also stimulates an increase in both hepatocyte growth factor expression and syndecan-1 shedding to enhance hepatocyte growth factor signaling, resulting in an aggressive phenotype [37].

In addition to modulating ECM rearrangement and growth factor signaling at the cell surface, heparanase also regulates the level of syndecan-1 in the nucleus [38]. Increasing expression of heparanase by myeloma cells drastically decreases the levels of syndecan-1 in the nucleus. Nuclear syndecan-1 is involved in several functions, such as controlling cell proliferation [39], gene transcription via inhibition of DNA topoisomerase 1 [40] and histone acetyl transferase activity [41]. Therefore, loss of nuclear syndecan-1 results in up-regulation of genes involved in tumor progression.

The transport of syndecan-1 and FGF2 to the nucleus is not completely understood, but studies have shown that shuttling of FGF2 and probably other heparin-binding growth factors to the nucleus is mediated by syndecan-1 via a mechanism involving protein kinase Cα and binding of syndecan-1 to tubulin [42,43]. Treatment with phorbol 12-myristate-13-acetate, under conditions that result in down-regulation of protein kinase Cα, decreases nuclear FGF2 levels [42]. Similarly, expression of a truncated version of syndecan-1, containing only the tubulin-binding sequence, is sufficient for nuclear translocation of FGF2, whereas removal of this sequence completely abolishes translocation [43]. In several cell types, nuclear targeting of FGF2 requires binding to its receptor FGFR1. Interestingly, in mesothelioma cells, although syndecan-1 and FGF2 are physically associated in the nucleus, FGFR1 is not detected in this subcellular compartment. This suggests that the syndecan-1/FGF2/FGFR1 complex is disassembled after internalization. The syndecan-1/FGF2 complex follows its microtubule-guided pathway to the nucleus, probably inside endosomes, whereas FGFR1 follows specific routes to the lysosome to be recycled. The presence in the nucleus of the syndecan-1/FGF2/heparanase complex suggests the existence of a nuclear heparanase-mediated regulatory mechanism for the turnover of heparan sulfate [44]. The nuclear transport of syndecan-1 and FGF2 to the nucleus and its effects on gene expression are summarized in Fig. 2.

As indicated by ^{35}S-sulfate incorporation in hepatocyte cells, nuclear translocation of HSPGs appears to occur after their secretion by the cells. In other words, they are not directly delivered from the Golgi to the nucleus [45,46]. The nuclear fate of extracellular HSPG appears to depend on the type of associated ECM proteins. It has been shown that the amount of nuclear HSPGs in stromal fibroblasts is higher when the cells are cultured on a fibronectin matrix as opposed to a collagen matrix, as the heparin-binding domain of fibronectin is required for the nuclear translocation [46]. Additionally, the HS chains of nuclear HSPGs differ from the rest of the intracellular pool. Nuclear HS chains are enriched in glucuronic acid 2-*O*-sulfated disaccharide units, suggesting that specific structure of HS may be required for the regulation of nuclear activities.

The expression of human sulfatase-1 (HSulf-1), a heparin-degrading endosulfatase, is diminished or even completely abolished in ovarian, breast, pancreatic and hepatocellular cell lines compared with normal tissues. This down-regulated HSulf-1 expression modulates a novel mechanism in cancer cells, promoting HS-dependent growth factor signaling [47]. Lower expression of HSulf-1 in breast cancer cells, under a hypoxic microenvironment, increases FGF2-induced signaling, cell migration and invasion [48], as well as autocrine activation of the EGFR/extracellular signal-regulated kinase (ERK) pathway leading to tumor growth [49]. In addition, ectopic over-expression of HSulf-1 in gastric cancer may function as a negative regulator of proliferation and invasion by suppressing Wnt/β-catenin signaling at the cell surface [50]. On the other hand, another human sulfatase enzyme, HSulf-2, an HS 6-*O*-endosulfatase, was found to inhibit *in vivo* tumor growth in breast cancer and to promote lung cancer through several signaling pathways, including Wnt [51,52]. Furthermore, this enzyme was found to exert an anti-apoptotic effect in hepatocellular carcinomas through activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway [53]. The importance of enzymes involved in HS metabolism withreagrd to cancer progression is reinforced by a study showing that exostosin (EXT) enzymes responsible for elongation of HS chains have been postulated to be tumor suppressors, preventing progression of osteochondromas to chondrosarcomas [54]. Furthermore, a recent study revealed that knockdown of EXT1 down-regulates the growth and survival of multiple myeloma cells via alteration of HS chains of syndecan-1 [55].

Structural modifications of cancer HS chains confer adaptive protection to malignant cells against natural killer cells, as the latter can only recognize transformed

cells exhibiting certain 6-*O*-sulfation and *N*-acetylation patterns. Pancreatic cells that express high levels of heparanase and HSulf activity avoid recognition by natural killer cells [56].

HS-based pharmacological approaches and future prospects

The results of studies investigating the importance of HS in molecular cancer cell mechanisms have led to development of HS-specific disease treatment. Several strategies have been utilized in order to achieve this purpose. A diagrammatic representation summarizing the various levels of pharmacological targeting in cancer is given in Fig. 1. One main target for HS-based therapy is inhibition of the activity of HS-degrading enzymes. Heparin, a polyanionic, linear, highly sulfated glycosaminoglycan, possesses 'natural' anti-cancer properties in addition to its potency as an anticoagulant [57]. Heparin species inhibit heparanase activity and binding to P- and L-selectins, suppressing the inflammatory process [58,59]. Examples of such agents are heparin, heparin-mimicking compounds, unfractionated heparin, low-molecular-weight heparin and ultra-low-molecular-weight heparin, which may act as potential metastatic inhibitors in cancer patients with thromboembolic complications. PI-88 (phosphomannopentaose sulfate), a recently developed heparanase inhibitor, is a highly sulfated oligosaccharide mixture currently undergoing phase II/III clinical trials in cancer patients with metastatic melanoma. This inhibitor also prevents angiogenesis and tumor growth by blocking specific interactions of FGF1, FGF2 and VEGF with their receptors and HS chains [60].

One drawback in the use of heparins as metastatic inhibitors is their anticoagulant properties, as long-term use of these molecules may lead to hemolysis and bleeding. Taking into account such side-effects, non-anticoagulant analogs such as a semi-synthetic sulfated hexasaccharide [61], a heparin-derived HS mimetic [62] and glycol-split heparin [63], which may inhibit heparanase and/or selectin activities *in vitro* and *in vivo* have been developed by chemical synthesis. Non-anti-coagulant low-molecular-weight heparins, such as dalteparin, are already in clinical trials for patients with small-cell and non-small-cell lung carcinoma, hormone-refractory prostate cancer and locally advanced pancreatic cancer [64]. In addition to already known semi-synthetic analogs or heparin mimics synthesized

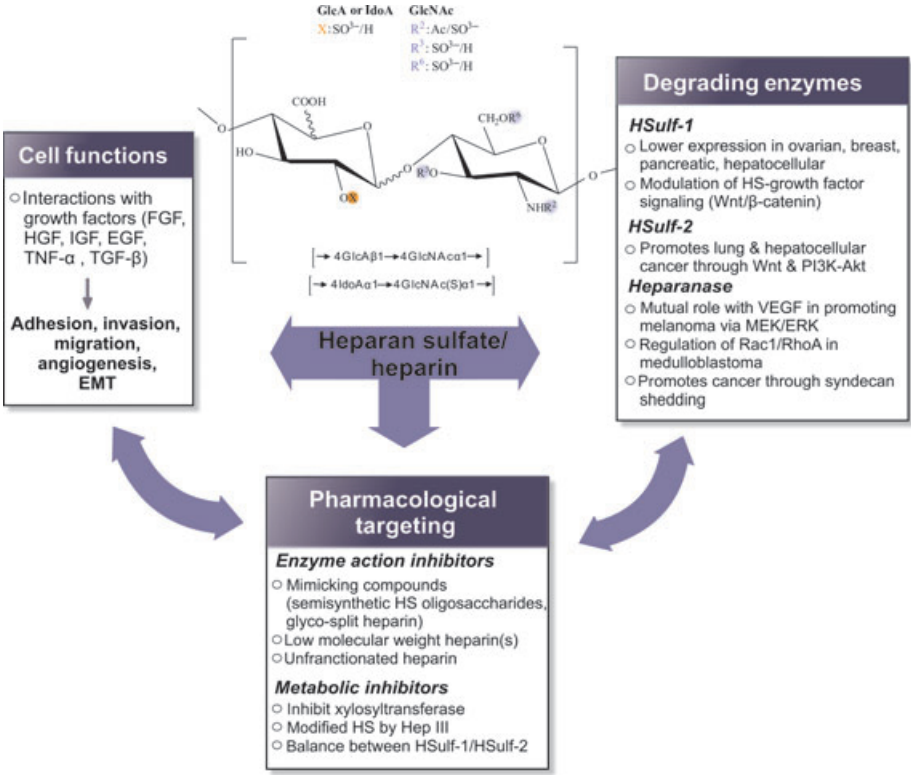

Fig. 1. Structure of the HS repeating unit, the properties of cancer cells that the HS chains may affect via interactions with growth factors (left), the main roles of HS degradation enzymes (right), and the level of pharmacological targeting (bottom).

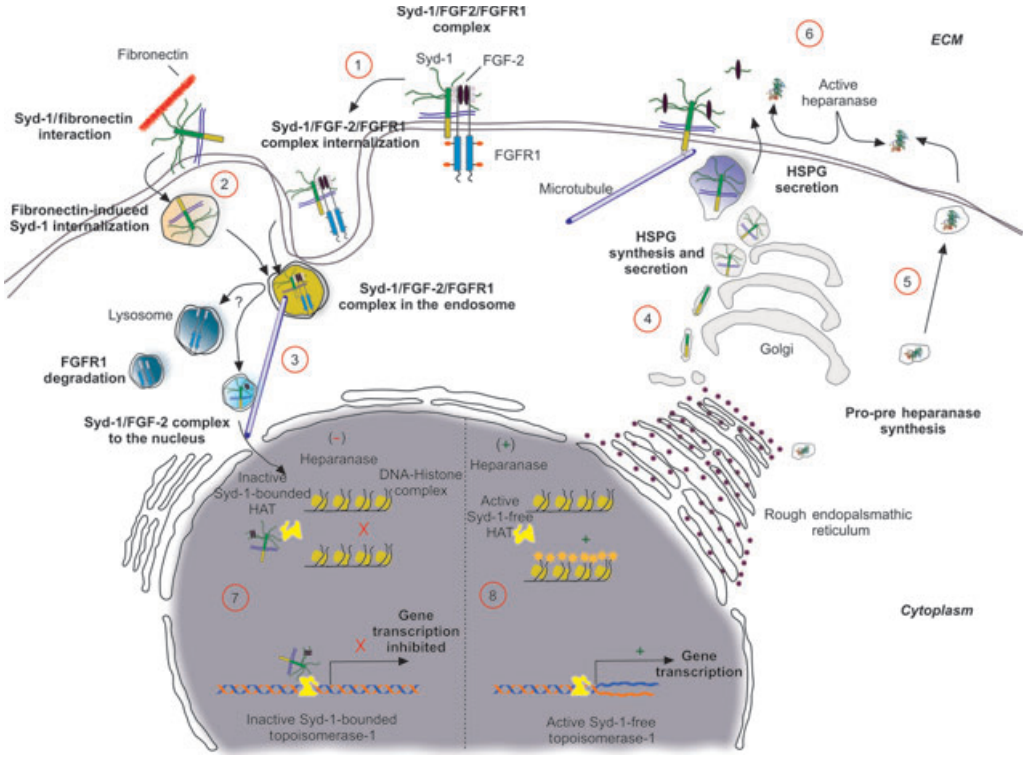

Fig. 2. Nuclear transport of syndecan-1 (Syd-1) and FGF2 to the nucleus and its effect on gene expression. (1) Syd-1/FGF2 internalization is mediated by FGF2 binding to its receptor at the cell surface. (2) Extracellular binding of Syd-1 to fibronectin at the ECM can also induce its internalization. (3) Once inside the cell, the Syd-1/FGF2 complex but not FGFR1 is translocated to the nucleus, probably inside endosome vesicles guided by microtubules. FGFR1 may follow a degradation pathway to the lysosome. Syd-1 does not follow a direct nuclear route from the Golgi. It needs to be secreted and endocytosed (1) (2) (4). Expressed and secreted heparanase (5) acts at the cell surface with ECM-secreted HSPGs (6) and induces loss of nuclear Syd-1 (8), allowing gene transcription. The presence of Syd-1 in the nucleus inhibits histone acetyl transferase (HAT) and topoisomerase 1, inhibiting gene transcription (7).

by chemical synthetic approaches, recent studies have described a new chemoenzymatic preparation of bioengineered ultra-low-molecular-weight heparin [65]. As highlighted by Lindhardt and Liu [66], chemoenzymatically produced ultra-low-molecular-weight heparin, in addition to maintaining the pharmacological properties of the synthetic chemical, it has a shorter and more efficient synthesis process. Improvement of the synthesis enzymes, such as sulfotransferase co-factor recycling and sulfo group donor 3′-phosphoadenosine-5′-phosphosulfate, will play a significant role in generation of synthetic heparin [67].

Other HS-dependent treatments for cancer therapy involve proteins or peptides with positively charged amino acid residues that antagonize heparin or HS chains by binding to their negative sulfated groups [68,69]. An example is suramin, which has the ability to occupy the heparin-binding sites of proteins, and is undergoing phase I clinical trials [70,71]. Metabolic inhibitors are another novel therapeutic strategy: for example, various xylosides have been used as competitive inhibitors for xylosyltransferase that initiate polysaccharide substitutions on core proteins [2, 16].

Modification and neutralization of HS functions may also be a potential therapy. For example, modification of HS with bacterial heparitinase III generated biologically active HS fragments that inhibit tumor cell growth and metastasis [72]. As HSulf-1 is down-regulated in human cancers, a recent promising therapeutic strategy is to restore the expression of this enzyme by using a recombinant plasmid DNA carrying HSulf-1 cDNA (pHSulf-1) delivered by heparin/polyethyleneimine nanogels [73]. Targeting individual HSulfs or HSulf-1/HSulf-2 interactions may constitute a novel pharmacological intervention in various cancer types.

Over recent decades, detailed structure characterization of specific HS motifs related to certain biological functions has encountered problems due to lack of useful tools and the extensive heterogeneity of HS chains. The development of phage display technology for generation of antibodies against HS enabled a novel and innovative approach. Utilization of these specific

antibodies has enabled clarification of several HS-dependent biological functions, and their specific action may be of utmost importance for detection of polysaccharide biomarkers in several diseases [74,75]. Phage display technology led to development of the first antibody against a determined sequence HS motif. This antibody (NS4F5) recognizes a highly sulfated HS motif $(GlcNS6S\text{-}IdoA2S)_3$. This HS motif is up-regulated in ovarian cancer, and NS4F5 antibody may be a potent agent for evaluation of this HS motif as possible biomarker. It may also have therapeutic applications [76]. Cell-surface HSPGs are important in cellular binding and uptake of polyamines, molecules that coordinate cancer cell proliferation. Inhibition of HS chains using a single-chain variable-fragment antibody against HS exploits the ability of the antibody to inhibit polyamine binding and internalization and therefore polyamine-dependent proliferation in several types of cancer cells [77].

CS/DS in cancer progression and cell signaling

CS/DSPGs: structure and function

CS is composed of repeating disaccharides of *N*-acetylgalactosamine and D-glucuronic acid (or L-iduronic acid in the case of DS), with various sulfation patterns (Fig. 3). Advances in analytical tools have shown that sulfation of CS/DS units may result in 22 differently sulfated disaccharide units [78,79]. Based on their major sulfation patterns, CS chains are classified as CS-A [GlcA-GalNAc-4-sulfate] or CS-C [GlcA-GalNAc-6-*O*-sulfate]. Furthermore, based on to the presence of disulfated units, CS chains may also be classified as CS-D [GlcA(2-*O*-sulfate)–GalNAc(6-*O*-sulfate) or CS-E [GlcA-GalNAc-(4,6)-*O*-disulfate]. DS, formerly designated CS-B, is characterized by the presence of IdoA moieties. Sulfation patterns of IdoA-containing units may involve IdoA(2-*O*-sulfate) and/or GalNAc(4-*O*-sulfate and/or 6-*O*-sulfate). The multiple sulfation motifs on C4 and/or C6 of *N*-acetylgalactosamine and/or C2 of hexuronic acid enable specific interactions with various molecules, including growth factors, cytokines, chemokines, adhesion molecules and lipoproteins [80].

PGs containing CS/DS chains are located in the extracellular space (aggrecan, versican, etc.), at the cell membrane (syndecans and glypicans) and also intracellularly (serglycin). Up- or down-regulation of expression of chondroitin sulfate PGs (CSPGs) is correlated with both normal and pathological conditions. CSPGs are able to regulate key cellular processes, including proliferation, apoptosis, migration, adhesion and inva-

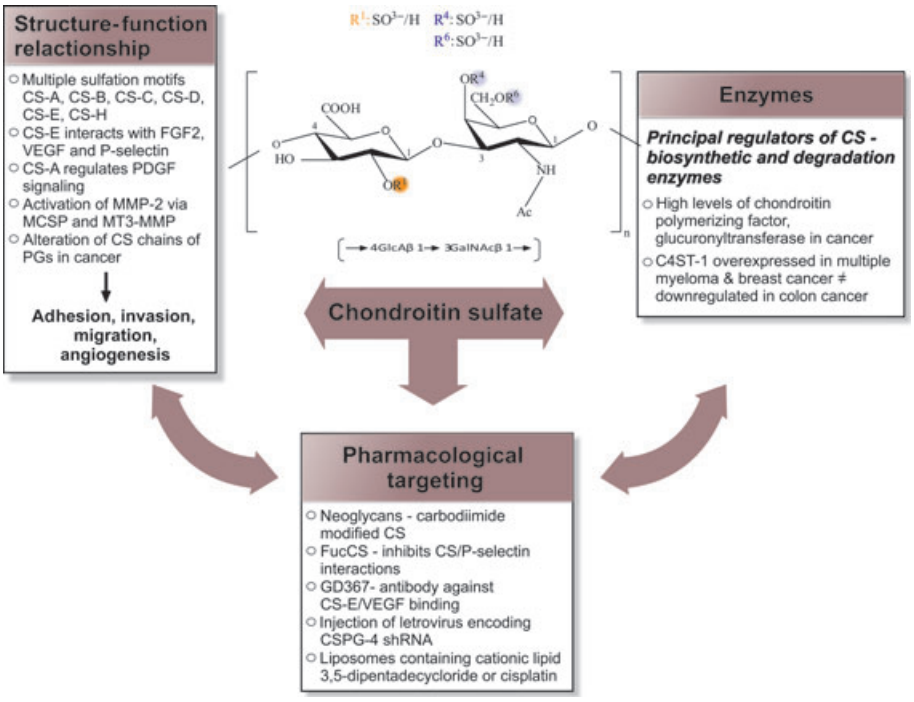

Fig. 3. Structure and contribution of CS to cancer progression. The structure of the CS repeating units is shown in the center, the various properties of cancer cells that the CS may affect via interactions with growth factors are shown on the left, the main enzymes involved in CS biosynthesis and degradation are shown on the right, and the level of pharmacological targeting is shown at the bottom.

sion, as well as ECM assembly, via their highly negative charged CS/DS side chains [80]. Versican and decorin are the major CSPGs, and are over-expressed in the stroma of a wide variety of malignant tumors, including osteosarcoma, testicular tumors, breast, pancreatic and colon cancer [81–83].

The importance of CS sulfation in growth factor-mediated signaling and cancer progression

Structural alterations of CS chains in CSPGs result in various activities with respect to growth factors and their receptors, enabling them to specifically promote various biological effects. Furthermore, the specificity of CS interactions with their ligands appears to be distinct from that of HS.

The type, i.e. sulfation pattern, of CS appears to be a critical factor in cancer progression [81]. CS chains interact with growth factors or store them in the ECM and release them gradually in the matrix, promoting cell signaling. For example, CS-E and CS-A chains bind to heparin-binding factors such as FGF2 and create triple complexes with their receptors to regulate their activation, which ultimately results in promotion of signaling pathways related to tumor progression [18,84]. High expression of CS-E in the ECM of ovarian adenocarcinomas is capable of mediating VEGF binding, in contrast with normal ovary and cystadenomas, in which CS-E is not expressed [85]. Structural analysis of CS/DS chains from two mouse Lewis lung carcinoma cell lines revealed a higher proportion of $\Delta^{4,5}$HexA-GalNAc-4,6-*O*-disulfate generated from CS-E in the highly metastatic cell line compared to the cell line with low metastatic potential [86]. Similarly, in murine LM8G7 osteosarcoma cells, which form tumor nodules in liver, expression of CS-E units is higher than in non-metastatic LM8 cells [87]. Moreover, colonization of liver by osteosarcoma cells was inhibited by pre-incubation of the metastatic LM8G7 cells with GD3G7 antibody against CS-E or by CS-E pre-administration [87]. Sulfation patterns also play a key role in the interactions mediated by selectins and their ligands. Specifically, DS and CS-E bind to L- and P-selectin, whereas CS-A, DS containing IdoA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate and/or 6-*O*-sulfate), CS-C, CS-D and CS-E bind to CD44 receptor [80,88,89]. In this regard, the sulfation pattern is an important factor in cancer progression correlated with selectin expression.

Metalloproteinases (MMPs) are zinc-dependent endopeptidases and the principal degradation enzymes contributing to cancer progression [90]. MMP downstream signaling, which is also correlated with malignant transformation, is affected by the type of CS sulfation. Thus, in melanoma cells, the CS-A sulfation pattern specifically enhanced formation of a three-molecule complex consisting of melanoma chondroitin sulfate proteoglycans, membrane type 3 MMP (MT3-MMP) and pro-MMP2, leading to activation of MMP2, a principal degradation enzyme in the metastatic cascade [91]. Furthermore, expression of melanoma chondroitin sulfate proteoglycans in melanoma cells enhances integrin functions and constitutive activation of the ERK1/2 pathway, stimulating cell growth and motility [92].

Exogenous CS chains also appear to regulate crucial cell signaling pathways involved in cancer pathogenesis. Intriguingly, signaling by platelet-derived growth factor BB (PDGF-BB) is affected by CS-A treatment of both normal and transformed cells of mesenchymatic origin. A significant co-stimulatory effect of CS-A in combination with PDGF-BB on the growth of HT1080 and B6FS human fibrosarcoma cells was demonstrated by Fthenou *et al*. [93]. This effect was not due to transcriptional up-regulation of PDGF receptor (PDGFR) genes, but instead to more efficient signaling by their protein tyrosine kinase domains [93]. On the other hand, both soluble and surface-bound CS-A down-regulate the mitogenic responses of PDGF-BB in normal human lung fibroblasts through reduction of PDGFRβ phosphorylation at tyrosine residue 1021, which is known to be specific for receptor activation [94]. Therefore, it appears that CS-A regulates PDGFR activation among normal and cancer cells in a manner depending on PDGF-BB presentation. Furthermore, exogenous CS-A affects key intracellular signaling pathways, including that of mitogen-activated protein kinases, to regulate fibrosarcoma cell adhesion, motility and migration [95]. Moreover, it has been shown that treatment with exogenous CS-B (DS) inhibits cell proliferation of both normal and malignant cells of osteoblastic origin [96]. These studies collectively suggest that CS-dependent signaling participates in mesenchymal tumor development [97] (Fig. 4).

The GAG chains of CSPGs participate in various types of interactions within the ECM, and consequently their absence or presence is of particular importance in malignancy. Thus, versican is over-expressed in melanoma cells, promoting metastasis, and has also been correlated with poor prognosis and cancer relapse in breast and prostate cancers [98,99]. Structural alterations of CS chains bound to versican and decorin have been identified in pancreatic, rectal and gastric carcinomas, in which expression of chains predominantly consisting of 6-*O*-sulfated and non-sulfated disaccharide units was enhanced [81,100,101]. In

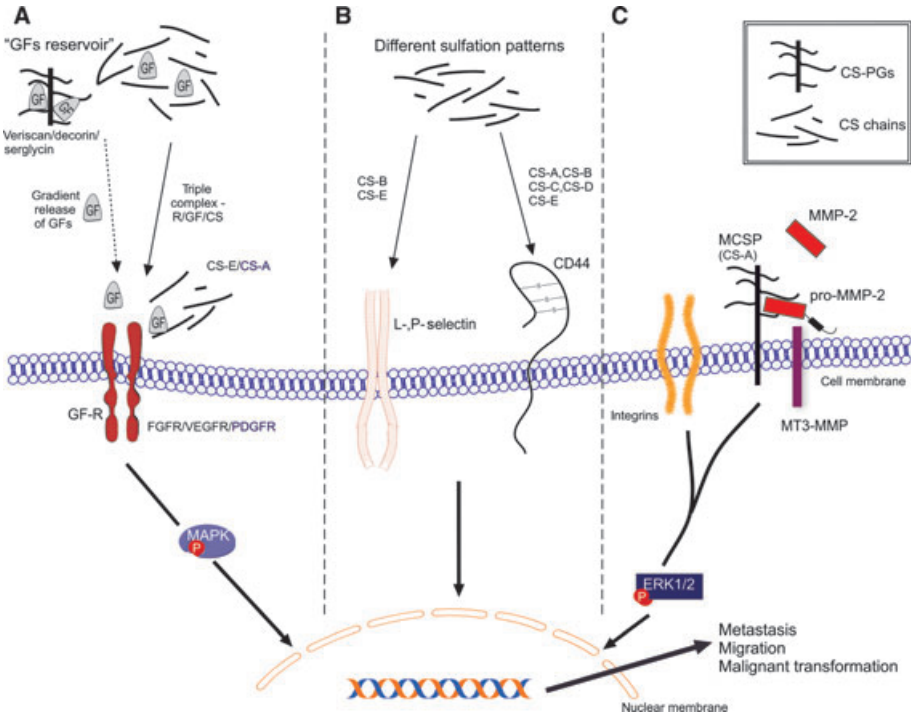

Fig. 4. CS-mediated signaling pathways and biological actions. The CS chains shown are either components of CSPGs or exogenously added free chains bearing various sulfation patterns. (A) Release of growth factors (GFs) from CS chains or delivery of GFs through CS chains creating a triple complex with receptors of growth factors (GF-R), leading to activation of the mitogen-activated protein kinase (MAPK) pathway and malignant transformation. (B) The importance of the CS sulfation pattern in interactions with cell membrane receptors such as L- and P-selectins and CD44. (C) Specific role of CS-A chains of melanoma chondroitin sulfate proteoglycans (MCSP) in activation of MMP2 through a triple complex with pro-MMP2 and MT3-MMP, promoting metastasis.

addition, some versican isoforms are up-regulated by TGFβ, resulting in the aggressive behavior of human osteosarcoma cells [102]. In contrast, the CS chains of decorin, a small leucine-rich proteoglycan, appear to exhibit anti-cancer properties independently of its protein core [103], with the exception of human osteosarcoma MG-63 cells [104]. Serglycin, which contains CS-A chains composed of up to 93% 4-sulfated disaccharides, is constitutively secreted by multiple myeloma cells, but is also localized on the cell surface via the CS-A chains [105]. Serglycin inhibits bone formation and activity of the classical and lectin pathways of the complement system, protecting multiple myeloma cells from immune system attack [105,106].

The biosynthetic and degradation enzymes involved in metabolism of CS are the principal regulators of the CS chain profile in a malignant environment. For example, in colorectal cancer, chondroitin-polymerizing factor, glucuronyltransferase and chondroitin synthase III are expressed at much higher levels in adenomas compared to adjacent normal tissue, and their expression gradually increases with cancer stage. The opposite expression profile was obtained for chondroitin synthase I [107]. The increased expression of chondroitin 4-sulfotransferase 1 in malignant cells of multiple myeloma as well as human breast cancer suggests a potential facilitating role in progression of these cancer types [108,109]. However, chondroitin 4-sulfotransferase 1 expression appeared to decrease with increased stages of colorectal cancer, and this requires further examination to determine its biological significance [107].

CS-based targeting in cancer

The role of CS as pharmacological agent may be achieved via direct uptake or as part of drug delivery system targeting cancer. Figure 5 summarizes the various levels of CS-based targeting in cancer.

Modified molecules called neoglycans are able to promote apoptosis of multiple myeloma cells. These carbodiimide-modified CS chains were demonstrated to reduce or even abolish tumor growth when directly injected in nude mice with breast cancer without causing toxicity [110]. The interactions of CS chains with P-selectins induce metastasis, based on their sulfation

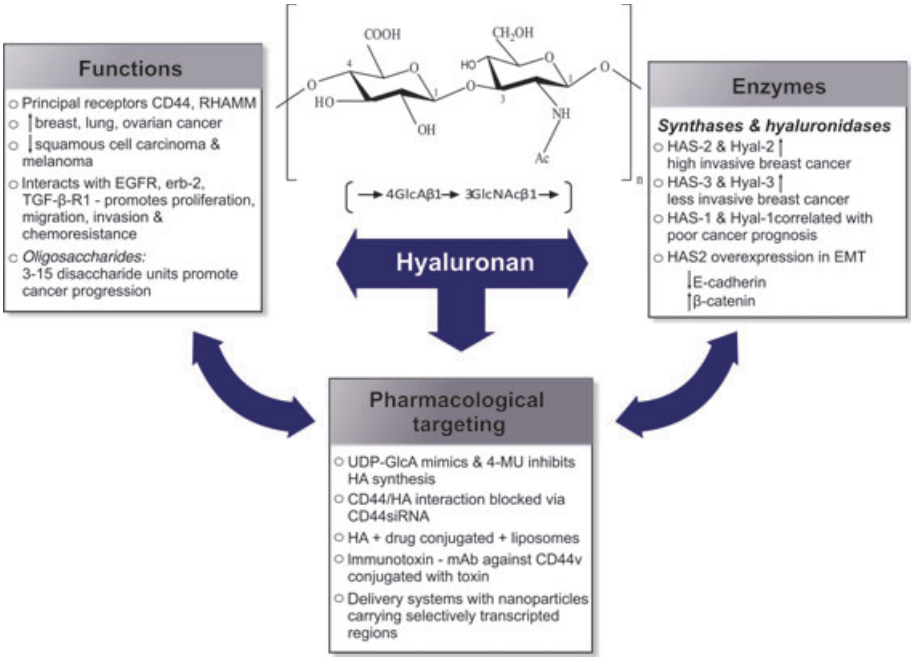

Fig. 5. Structure of the HA repeating unit, the various properties of cancer cells that the HA chains may affect via interactions with growth factors (left), the main roles of HA biosynthetic and degrading enzymes (right), and the level of pharmacological targeting (bottom).

profile. Over-sulfated CS chains and a fucosylated CS isolated from sea cucumber have an inhibitory effect on P-selectin-mediated events [111,112]. Moreover, in ovarian adenocarcinomas, the antibody GD3G7 raised against a specific CS-E sequence competitively inhibited CS-E binding to VEGF, suppressing tumor progression [113]. It has been shown that CS-B (DS) is highly expressed in tumor stroma and rarely in tumor cells. Thus, a specific phage display-derived antibody GD3A12 that recognize DS with a high content of IdoA-GalNAc4S disaccharide units may be a novel useful tool against disease progression [114]. CSPG4 is a regulatory molecule in melanoma cell growth and metastasis. Its expression may be inhibited using monoclonal antibody against CSPG or by direct injection of a lentivirus containing short shRNA encoding CSPG4 [115]. Another promising approach concerning CS targeting is the design of monoclonal antibodies against specific CS sequences (CS-0S, CS-4S and CS-6S) of CSPGs (e.g. aggrecan), blocking their action [116,117].

Discrete CS expression in cancer tissues may be utilized when releasing in gradient liposomes carrying anti-cancer drugs to target malignant cells or tissues. Thus, the over-expression of CS in several metastatic tumors enables selective delivery of anti-cancer drugs by polyethylene glycol-coated liposomes. In particular, liposomes containing the cationic lipid 3,5-dipentadecycloxybenzamidine hydrochloride are rapidly internalized by tumor cells when bound preferentially to CS. Loading these liposomes with cisplatin enables the inhibitory action of this chemotherapeutic agent to selectively affect tumor cells over-expressing CS [118]. CS is also used for gene delivery systems via polyethyleneimine nanostructures that are designed to selectively target cancer cells by utilizing the ability of polyethyleneimine to interact with specific cell-surface receptors [119]. Moreover, use of biodegradable polymers including CS has been proposed as a colon-specific oral drug delivery system [120]. When cross-linked with chitosan, CS selectively releases orally administered indomethacin to the colon [121]. Furthermore, initial progress has been made in exploiting the discrete CS expression of malignant tissues as a diagnostic tool. Thus, a low degree of CS sulfation has been utilized as a specific marker in follow-up of patients with head and neck tumors [122]. Further structural elucidation of CS/DS functional domains is required in order to obtain information for the development of new therapeutic strategies.

Hyaluronan

HA structure and function

HA is a linear non-sulfated glycosaminoglycan consisting of repeating disaccharides of D-glucuronic and *N*-acetylglucosamine with a wide range of molecular

weights from 10^5 to 10^7 Da (Fig. 5). HA is produced by three HA synthase isoenzymes (HAS1, HAS2 and HAS3), which are integral plasma membrane proteins whose active sites are located at the intracellular face of the membrane [123]. Each of these three enzymes polymerizes a different size of HA chains. HAS1 activation leads to production of high-molecular-weight HA (2000 kDa), but its synthetic activity is lower in comparison with the other two enzymes. HA of similar molecular weight is also produced by HAS2, which furthermore is implicated in developmental processes involving tissue expansion and growth. HAS3 has the highest activity and produces shorter HA chains with molecular weights between 100 and 1000 kDa.

HA synthase activity is regulated by various factors, such as growth factors, protein kinase C activators and cytokines. Pro-inflammatory factors such as interleukin-1β, TNFα and TNFβ induce HA synthesis, whereas TGFα and TGFβ have the opposite effect [124]. When the AMP concentration increases, AMP-activated protein kinase is activated and induces glucose and fatty acid catabolism through phosphorylation of specific enzymes that participate in the metabolic pathways. It has been also found that, under treatment with anti-hyperglycemic drugs such as metformin or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, activation of AMP-activated protein kinase through phosphorylation is more intense [125]. Recently, it was found that AMP-activated protein kinase phosphorylates human HAS2 at Thr110, inhibiting its enzymatic activity in human aortic smooth muscle cells. This effect was stronger when the cells were treated with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside or metformin. In contrast, HAS1 and HAS3 are not modified by the kinase [126]. HAS2 is also modified by mono-ubiquitination on Lys190, which has been demonstrated to be required for HAS2 activity [127] (Fig. 6). In particular, COS-1 cells were transient or stable transfected with Flag- or 6myc-HAS2 and total cell lysate as well as anti-Flag- or anti-Myc-immunoprecipitated material were subjected to SDS/PAGE followed by immunoblot analysis for the tag-HAS2 and ubiquitin. Furthermore, the identification of both HAS2 protein and its mono-ubiquitination on Lys190 was achieved by MALDI-TOF analysis. A K190R mutant of HAS2 showed reduced mono-ubiquitination and a strong decrease in HA synthesis [127].

Newly synthesized HA is located at the cell surface, in pericellular and extracellular matrices, or even attached to HAS, interacting with cell-surface receptors. HA is also present in intracellular compartments. A significant amount of HA is present in tissue regions characterized by high cell division and invasion capabilities [128]. HA may also act as a signaling mediator via binding with HA receptors such as CD44, the hyaluronan-mediated motility receptor RHAMM, Toll-like receptors 2 and 4, lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and hyaluronan receptor for endocytosis (HARE)/stabilin-2 [120,129–131]. Recently, CD44 and the hyaluronan-mediated motility receptor RHAMM have been defined as major receptors implicated in cancer progression. Degradation of HA is achieved through the enzymatic action of hyaluronidases (Hyal-1, Hyal-2, Hyal-3 and PH-20), whose expression is increased or suppressed in some malignant conditions compared with normal tissues. Hyal-1 and Hyal-2 are the main hyaluronidases involved in HA catabolism and act in cooperation. First, Hyal-2 degrades high-molecular-weight HA on the cell surface to variously sized HA fragments, which, after endocytosis, are degraded intracellularly by Hyal-1 to tetrasaccharide residues. It is believed that the various oligosaccharide fragments have distinct effects in cancer progression, angiogenesis and metastasis [132].

HA in cancer biology

It is well documented that the tumor microenvironment and the growth factors and chemokines produced therein induce HA production [133]. The presence of HA is correlated with malignancy in many types of human cancer. The functions of HA in cancer progression vary according to its size and amount in the tumor stroma. HA accumulates to high levels in adenocarcinomas such as breast [134], lung [135] and ovarian cancer [136]. In contrast, the HA content is low in squamous cell carcinoma and melanoma, and its absence is directly correlated with the metastatic potential [137,138]. An extracellular complex of versican with HA and CD44 creates a viscoelastic environment in the pericellular matrix, promoting cancer cell proliferation and migration. High levels of standard CD44 (CD44s) and CD44 variants (CD44v) increased synthesis of HA [139]. CD44 in HA-rich ECM interacts directly or indirectly with signaling receptors such as EGFR, ErbB2 and TGFβ receptor type I, and subsequently stimulates downstream signaling pathways such as PI3K/Akt or mitogen-activated protein kinases, promoting chemoresistance and cancer progression [129,140–142].

The gene expression profile of HA metabolic enzymes is altered during invasion and metastasis. In breast cancer, the more invasive cancer cells express HAS2 and Hyal-2 isoforms, while the less invasive cells produce more HAS3 and Hyal-3 [143]. HAS1 was

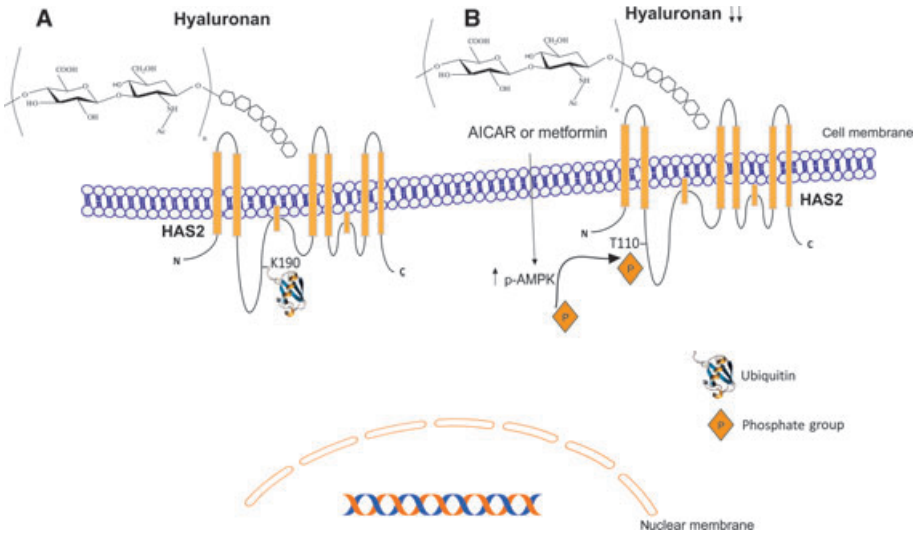

Fig. 6. Post-translational modification of the membrane enzyme HAS2. (A) Synthesis of hyaluronan requires mono-ubiquitination at K190. The lysine residue at position 190 is located in one of the cytoplasmic domains of HAS2, where the glycosyltransferase activity is predicted to reside. (B) In the same cytoplasmic domain, phosphorylation of threonine 110 by AMP-activated protein kinase inhibits the enzymatic activity of HAS2. AMPK phosphorylation is activated by two drugs: metformin and 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR).

detected in multiple myeloma patients and is associated with poor survival of patients [144]. In colon carcinomas, over-expression of Hyal-1 suppresses tumorigenesis [145]. It has been shown that, depending on its concentration, Hyal-1 functions as a tumor promoter or a tumor suppressor [146,147]. CD44 cleavage has been also reported in correlation with tumor progression, such as breast, ovarian, glioma, non-small cell lung and colon cancers [148]. This effect is attributed to the variously sized HA fragments. In general, hyaluronidases in the tumor microenvironment result in partial or complete degradation of HA. These HA oligosaccharides induce cleavage of CD44, promoting cancer cell motility and invasion [149]. Furthermore, HA oligosaccharides possess angiogenic properties depending on their molecular size. HA fragments of 3-15 disaccharide units are able to stimulate endothelial cell proliferation, adhesion and capillary formation [150,151]. High levels of these fragments have been detected in squamous carcinomas of the head and neck area [152]. Moreover, HA oligosaccharides disrupt the interactions between the high-molecular-weight HA and its receptors on tumor cells [153], inhibiting tumor growth *in vitro* and *in vivo* [154] in a manner that is at least partly dependent on suppression of the PI3K/Akt pathway [155]. In addition, the tetrasaccharides exhibit anti-apoptotic actions as they inhibit cell death under stress conditions [156]. It has also been reported that HA oligosaccharides inhibit progression of bone metastasis in breast cancer by abolishing HA–CD44 interactions [157].

HA plays an important role in the process of epithelial/mesenchymal transition, during which the cells lose their epithelial formation establishing a migratory phenotype. The mesenchymal phenotype is important in embryonic development and is an obligatory step of the metastatic cascade. The role of HA in organogenesis has been demonstrated in HAS2 null embryos, in which endocardial cushion cells fail to undergo epithelial/mesenchymal transition and do not migrate to the cardiac jelly, resulting in abnormal heart development [158]. Over-expression of HAS2 is correlated with down-regulation of E-cadherin, an epithelial marker, and β-catenin, supporting the view that HA over-production induces the epithelial/mesenchymal transition in cancer cells [159]. Moreover, the increased amount of HA promotes invasiveness by inducing MMP2 and MMP9 production via the PI3K/Akt pathway in MCF-10A human mammary epithelial cells.

An association between the development of cancer and inflammation has long been established [160,161]. There is evidence to suggest that long-term inflammation is highly associated with cancer metastasis. In inflammatory conditions, the well-documented increased production of stromal HA helps to create a highly hydrated ECM, thus facilitating local cellular trafficking [162,163].

HA targeting in cancer

One way to target HA is by inhibiting its synthesis and specifically by disabling the production of

UDP-α-D-glucuronic acid (UDP-GlcA) [164]. To date, novel therapeutic strategies have involved development of antagonists specific for UDP-α-D-glucose 6-dehydrogenase, the enzyme that oxidizes UDP-GlcA. A synthetic UDP-GlcA mimetic, UDP-7-deoxy-α-D-gluco-hept-6-ulopyranose, exhibited reversible inhibition of UDP-α-D-glucose 6-dehydrogenase [165] (Fig. 5). In addition, inhibition of human UDP-α-D-glucose 6-dehydrogenase by quercetin and the polyphenol, gallic acid, suppressed the proliferation of MCF-7 human breast cancer cells *in vitro* [166]. HA biosynthesis was also down-regulated by 4-methylumbelliferone in melanoma cells without any toxic effects [167,168]. Moreover, 4-methylumbelliferone suppressed HA retention and cell-associated matrix formation in osteosarcoma cells, resulting in a reduction of tumorigenicity [169]. Recent studies also demonstrated that 4-methylumbelliferone induces apoptosis of hepatic stellate cells *in vitro* and decreases the number of activated cells *in vivo* [170].

Another level of HA targeting involves inhibition of HA/CD44 interactions by using CD44 siRNA. This method is encouraging but faces difficulties concerning low levels of siRNA transfer efficiency or the short period of siRNA stability, restricting the duration of siRNA-induced phenotypic changes to short time periods. To overcome these difficulties, Misra *et al.* [171] developed a new model for shRNA delivery, targeting CD44v6 in colon cancer. This project utilized a non-viral vector, with plasmids packed in nanoparticles formed from a polyethylene glycol and polyethyleneimine layer coated inside and outside with transferrin. The transferrin binds to a specific transferrin receptor that is over-expressed in cancer cells. In the nanoparticles, the encapsulated shRNA plasmids contain a tissue-specific promoter-driven Cre recombinase and conditionally silenced plasmid. Successful delivery of the vector depends on transcription of Cre recombinase selectively in cancer cells.

Prevention of CD44–HA interactions can also be achieved by using antibodies targeting highly expressed CD44 variants. These antibodies can selectively deliver the drugs to CD44, leading to cell apoptosis [172]. To strengthen this anti-cancer action, the macromolecular CD44–HA carriers are delivered intravenously directly to the tumor location, enabling them to selectively extravasate into the tumor rather than healthy tissues. Exploitation of this concept resulted in the production of immunotoxin: an anti-CD44v6 antibody conjugated with a cytotoxin. This antibody against the variant form is coupled with the cytotoxic drug mertansine or radioisotopes, and selectively targets malignant cells [173]. This immunotoxin was subjected to phase I clinical trials; however, after an initial good response, it was withdrawn due to limited success [174]. Toxicity observed in clinical trials with anti-EGFR agents is correlated with high expression of CD44 receptors in normal tissues such as skin. HA is also used as both a drug carrier and ligand on liposomes or nanoparticles targeting tumor cells that over-express CD44. An additional property of HA–CD44 conjugates is internalization of the drug [174,175]. Thus, following release, activation of the conjugates is achieved upon intracellular enzymatic digestion [176,177]. In addition, the unique properties of HA may be exploited by conjugating this GAG to low-solubility drugs in order to provide better coating and structural protection, thus prolonging their bioavailability.

Concluding remarks

For many years, the ECM was considered to be an inert complex of macromolecules, but during the last decade many studies have elucidated the various valuable biological actions of matrix molecules, specifically glycosaminoglycans, in tissue development and homeostasis as well as pathological processes [178]. The interactions of GAGs with growth factors, cytokines and growth factors receptors are implicated in cancer growth and progression. The GAGs are involved in signaling cascades, regulating angiogenesis, invasion and metastasis of malignant cells. Understanding of the fine structure and specific biological roles of GAGs has led to novel therapeutic approaches, including development of HS mimics as well as delivery systems for anticancer drugs targeting over-expressed CS in the tumor microenvironment or HA–CD44 interactions. This is a fast growing and promising area of research for the design and development of novel powerful agents for diagnosis and prognosis, drug delivery and molecular targeted therapy in cancer.

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