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The role of heparan sulfate maturation in cancer: A focus on the 3O-sulfation and the enigmatic 3O-sulfotransferases (HS3STs)

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ABSTRACT

Heparansulfate (HS) modifications are master regulators of the cross-talk between cell and matrix and modulate the biological activity of an array of HS binding proteins, including growth factors and chemokines, morphogens and immunity cell receptors. This review will highlight the importance of HS maturation mediated by N-deactetylase/sulfotransferases, 2O- and 6O-sulfotransferases in cancer biology, and will focus on the 3O-sulfotransferases and on the terminal, rare 3O-sulfation, and their important but still enigmatic impact in cancer progression. The review will also discuss the molecular mechanisms of action of these HS modifications with regards to ligand interactions and signaling in the cancer process and their clinical significance.

1. Introduction

Heparan sulfate proteoglycans (HSPGs) are widely distributed macromolecules, present at the plasma membrane of virtually every cell and within extracellular matrices (ECM). They are also found intracellularly in mast cells granules [1] and targeted to the nucleus in some stromal and cancer cells [2]. They comprise a diverse group of glycoproteins containing one or several glycosaminoglycan (GAG)-HS chains covalently bound to a core protein [3]. The number of core protein genes is relatively limited and mammal HSPGs are classified in two major groups based on their location [4]: cell surface-bound HSPGs that comprise the glypican and syndecan families and the extracellular type mainly represented by perlecan and agrin. Most of the biological information carried by HSPGs lies in their HS chains, which are long, linear polysaccharides composed of disaccharide building blocks consisting of N-acetyl-D-glucosamine (GlcNAc) and an uronic acid (either a D-glucuronic acid (GlcA) or a L-iduronic acid (IdoA)). The disaccharide units are variably modified by N-deacetylation, N-, O-sulfation and epimerisation. HSPGs are predominantly assembled in the Golgi apparatus by a multistep non-template-driven process that involves more than 25 enzymes including glycosyltransferases (GTs) and maturation enzymes, mainly sulfotransferases (STs) [5,6]. These enzymes generate a unique molecular design with distinct functional regions, comprising hypervariable sulfated domains (S-domains) interspersed with low-sulfated N-acetylated regions (N-domains) [7]. Owing to the presence of sulfate groups at specific positions (N-, 2O-, 6O-, and 3O-), HS chains exhibit an overall highly negative charge and specific docking sites for a variety of interactors [8]. It has been proposed that sulfation generates a "HS code" whereby sulfated oligosaccharides encode functional information in a sequence-specific manner analogous to that of RNA, DNA and proteins [8,9]. In this regard, the 3O-sulfation represents a very important but still enigmatic HS modification since it forms HS binding motifs often recognized with a fine specificity and high affinity by effector proteins [10,11].

HS chains interact with a myriad of partners at the cell surface and within the ECM, including cell surface receptors, ECM proteins, enzymes, growth factors (GFs), cytokines and morphogens [12]. HS chains are also capable of binding to nuclear targets such as histones and histone modifying enzymes or transcription factors, potentially influencing the cell cycle and the cancer process [2,13]. Their large structural and functional diversity and the multiple interactions of HS with numerous ligands confer to HSPGs a tremendous variety of physiological roles. They participate to morphogenesis, wound healing, inflammation and immunity, and help to maintain cell and tissue homeostasis. Most of these biological processes are prone to be highjacked by tumor cells at each step of the cancer process and HSPGs strongly impact the cancer cell behavior, matrix remodeling and the progression of the malignancy. Thus, HSPGs are intensively scrutinized as actors and targets in the field of cancer glycobiology [14,15].

This field is evolving rapidly. Until recently, it was thought that

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HSPGs mainly act as major co-receptors for GFs, by concentrating them at the cell surface for high affinity binding and signaling. This paradigm is now shifting rapidly since more independent interactions occur at the cell surface and matrix interface, and with stromal and immunity cells that involve independent roles of HSPGs [16]. It is therefore important to examine more precisely the role of 3O-sulfation in view of the expanding repertoire of ligands interacting with 3O-sulfated HS chains including the paracrine Fibroblast Growth Factors FGF7 and FGF10 [17,18], the pleiotropic neuropilin (NRP) receptor [19,20] and immunity cells, such as Natural Killer (NK) cell receptors [19].

HS chains are now a topic of major interest in tumor biology and some achievements are summarized below. This review focuses on the sulfotransferases (STs) that are responsible for the major HS modifications and on the seven HS3ST isoforms that catalyze 3O-sulfation in particular, in the context of cancer. We shall review the pro- and anti-oncogenic roles of HS-maturation enzymes, their regulation, and how the specific sulfate arrangements that they produce are involved in key events driving diverse malignancies such as proliferation, angiogenesis, inflammation and immunity *via* pleiotropic ligand-HS interactions. A cancer-specific understanding of the HS interacting network could uncover potential mechanisms and therapeutic targets of these extraordinary multi-tasking biomolecules.

2. HS biosynthesis and maturation

HSPGs biosynthesis and maturation is a complex and coordinated process involving many GTs and STs which is finalized by the rare but crucial action of 3O-sulfotransferases (3OSTs, HS3STs) (Fig. 1). The process starts in the endoplasmic reticulum with the synthesis of the core protein containing specific serine residues, on which the GAG chains will be attached. The HS biosynthesis itself is initiated by the formation of a tetrasaccharide sequence [GlcA-β1,3-Gal-β1,3-Gal-β1,4-Xyl-O], generally referred to as the protein-GAG linkage region, which is common to HS/heparin (Hep) and chondroitin-/dermatan-sulfate PGs. This tetrasaccharide linker is synthesized by the successive and coordinated action of four GTs [21] : (i) xylosyltransferases I and/or II for the transfer of the first xylose residue [22], (ii) β1,4-galactosyltransferase-7 (β4GalT7) and (iii) β1,3-galactosyltransferase-6 (β3GalT6) which add the first and second galactose residues on xylose respectively [23,24] and finally (iv) the β1,3-glucuronosyltransferase-1 (GlcAT-1) [25] for the terminal addition of GlcA onto the linker. The xylose residue is modified by phosphorylation and the galactose residues by sulfation. Such modifications are thought to play a role in the regulation of initiation and the possible orientation of GAG synthesis [26,27]. HS chains are then polymerized onto this linkage region by the alternate addition of GlcNAc and GlcA residues thanks to the sequential activity of GTs from the exostosin-like (EXTL), in particular EXTL3, and the exostosin (EXT) families, EXT1/EXT2 [28,29].

Concomitantly with polymerization, HS maturation consists in further modifications of sugar units. They are mainly carried out by Golgi-associated STs, which catalyze the transfer of a sulfate group (from the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS)) onto the oligosaccharide growing chain. The first step of HS modification consists in the N-deacetylation of GlcNAc following by its N-sulfation, a key event in determining further modifications of polysaccharide chains. This step is catalyzed by glucosaminyl-*N*-deacetylase/N-sulfotransferase (NDST) family, which display both deacetylase and sulfotransferase activities [30,31]. Four NDST have been described (NDST1-4), which determine HS N-sulfation patterns and differently influence HS fine structure and functions. The second step of HS maturation involves 6O-sulfotransferases (HS6STs), responsible for a sulfate group attachment onto the C6 position of the glucosamine residues [32]. Then intervenes the D-glucuronyl C5-epimerase (Glce), which converts D-glucuronic acid to L-iduronic acid, thereby increasing HS structural diversity and chain flexibility [33,34]. 2O-sulfotransferases (HS2STs) catalyze the transfer of a sulfate group onto the 2-position of uronyl residues, mainly on iduronic acid (IdoA) residues [35], depending on the precise location of O-sulfate groups in the HS chain [36]. 2O-sulfated iduronic acid residues can be targeted by endogenous heparanases, underlying the important role of iduronic acid 2O-sulfation in HS catabolism and turnover [37]. A coordinated action of Glce and HS2ST in HS maturation has been suggested and physical interactions have also been demonstrated between Glce and HS2ST [38].

Finally, the terminal 3O-sulfation of previously N-sulfated glucosamine residues is catalyzed by the enzymes of the 3O-sulfotransferase family (3OST, HS3ST) [39]. 3O-sulfation is a rare event that depends on previous HS N- and O-sulfation rate at the vicinity of target motif. The target disaccharide [GlcNS,6S-IdoA2S], containing N- and 6O-sulfated glucosamine residue and 2O-sulfated uronic acid residue (mainly iduronic acid) next to the 3O-sulfation site, has been described as an efficient substrate for HS3STs [40,41]. Seven human HS3ST isoforms have been identified (HS3ST1, -2, 3A, -3B, -4, -5 and -6) with either ubiquitous or tissue specific expression. For example, HS3ST1, -2 and -4 display high expression in cerebellum and cerebral cortex whereas HS3ST3A, -3B and -6 are mostly expressed in liver [42,43] and HS3ST5 in skeletal muscle [44]. The tissue expression of HS3ST1, -2 and -3 (in cerebellum, placenta, spleen, stomach and small intestine among others) is larger than that of HS3ST6, the expression of which is restricted to liver and kidney [45]. Given the unusually low natural abundance of the 3O-sulfation, it is intriguing that as many as seven HS3ST isoforms have been identified in mammals, making them the largest family of HS maturation enzymes. Two types of HS3STs have been described with regards to 3O-sulfated HS interaction with specific ligands and with unique substrate specificity towards the disaccharide [GlcNS,6S-IdoA2S]: (i) the AT-type (HS3ST1 and -5), which generates specific binding site to antithrombin III (ATIII), leading to AT anticoagulant activity [46,47], and (ii) the gD-type (HS3ST2, -3, -4, -5 and -6) to create 3O-sulfated HS binding site to *Herpes simplex* viral envelope glycoprotein D (gD), which is used as an entry receptor for *Herpes simplex* virus 1 (HSV-1) and contribute to cell infection [48–50]. HS3ST1 (AT type) preferentially modifies a motif containing a 2O-sulfated GlcA at the non-reducing end of glucosamine [43,51,52]. This ST will tolerate unsulfated IdoA residue next to the target residue, but 2O-sulfation specifically prevents its action [41]. In contrast, HS3ST2, -3, -4 and -6 (gD type) preferentially sulfate oligosaccharide motives in which IdoA has been previously 2O-sulfated [42,45,53,54]. HS3ST5 modifies sites irrespective of uronic acid 2O-sulfation and consequently can produce both AT- and gD-type modifications [44,47]. 3O-sulfated HS motives (produced by HS3STs) constitute specific and high affinity binding sites for a series of ligands, controlling their biological activity in pathophysiological situations, as it will be discussed in the context of cancer in this review. Why the rare 3O-sulfation event is carried out by a large number of HS3ST isoforms remains a puzzling question. It may be speculated that the complex spatial distribution (between different tissues and possibly even different cell organelles as outlined below) and temporal expression of these isoforms have the potential to precisely control ligand binding and signaling in multiple tissues and in various pathological situations such as during development and cancer. In addition, it is worth mentioning that recent studies prompt re-evaluation of the natural abundance of HS 3O-sulfation that may be higher than generally considered and that consequently its functions may be broader [55]. Of note also, it has been suggested that the use of HS3ST during HS biosynthesis may not occur as the final enzymatic step of the pathway [56].

3. Role of HS sulfation in the generation of pro- and anti-oncogenic determinants

Since the synthesis and maturation of HS chains obey a non-template driven mechanism, the structure and sulfation pattern of HS chains mainly depend on the orchestrated action of STs. The variously sulfated motives that STs generate are involved in a vast array of

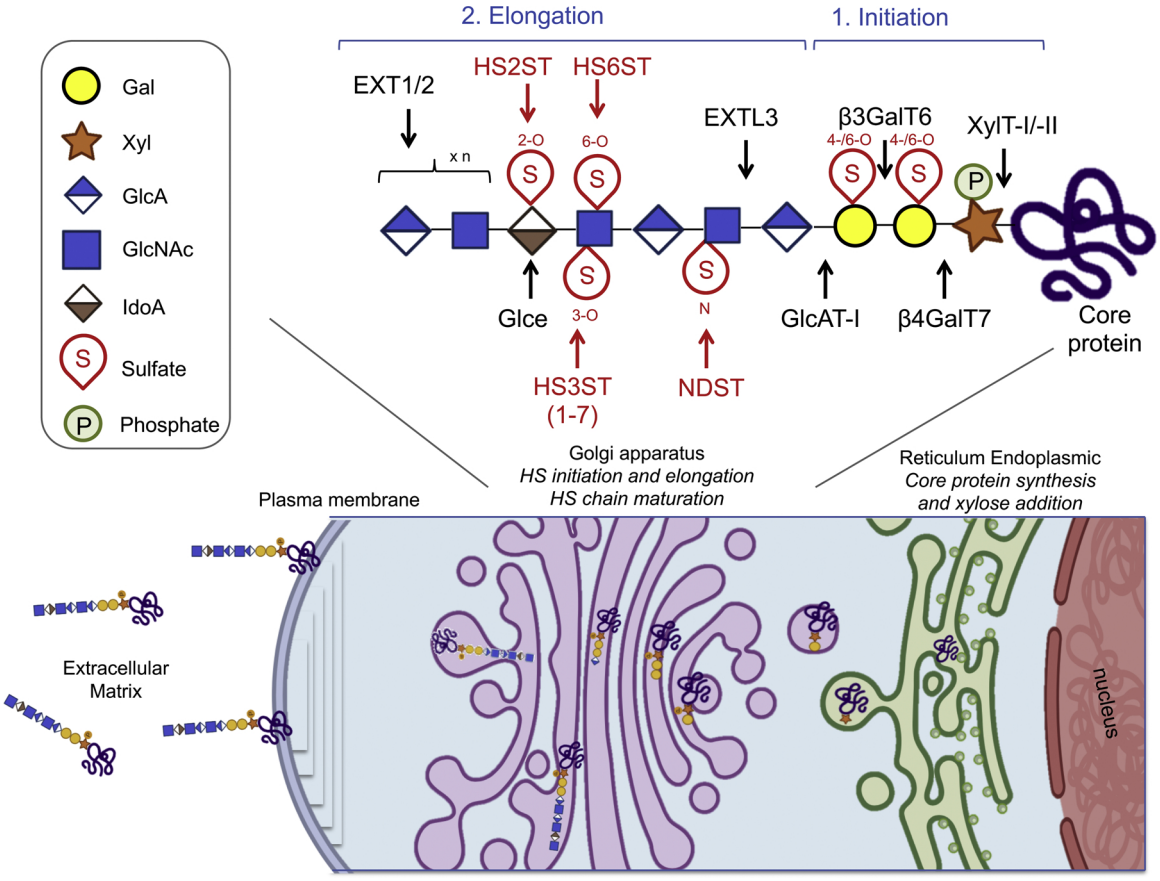

Fig. 1. Heparan Sulfate (HS) biosynthesis and maturation. Heparan Sulfate Proteoglycan (HSPG) biosynthesis and maturation involve many glycosyltransferases (GTs) and sulfotransferases (STs) which sequentially add sugar moieties in a coordinated manner. HSPG biosynthesis starts with the synthesis of the core protein in the endoplasmic reticulum. HS biosynthesis itself is then initiated by the formation of a tetrasaccharide protein-GAG linkage region [GlcA-β1,3-Gal-β1,3-Gal-β1,4-Xyl-O] attached to the core protein by (i) xylosyltransferases I and/or II (XylT-I/-II), (ii) β1,4-galactosyltransferase-7 (β4GalT7) and (iii) β1,3-galactosyltransferase-6 (β3GalT6), (iv) β1,3-glucuronosyltransferase-1 (GlcAT-1). HS chains are polymerized onto this linkage region by the alternate addition of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) residues by the sequential activity of GTs from the exostosin-like (EXTL), in particular EXTL3, and the exostosin (EXT) families, EXT1/EXT2. Finally, HS maturation consists in sugar modifications mainly carried out by Golgi-associated STs, from different families : (i) glucosaminyl-N-eacetylase/N-sulfotransferase (NDST) for GlcNAc N-deacetylation and N-sulfation, (ii) 6O-sulfotransferases (HS6STs), for a sulfate group attachment onto the C6 position of the previously modified glucosamine residues, (iii) D-glucuronyl C5-epimerase (Glce), which converts D-glucuronic acid to L-iduronic acid, (iv) 2O-sulfotransferases (HS2STs) for the transfer of a sulfate group onto the 2-position of uronyl residues (mainly iduronic acid (IdoA)), (v) 3O-sulfotransferases (HS3STs) for the 3O-sulfation of previously N-sulfated glucosamine residues. HSPGs are exported in the extracellular matrix or are anchored to the plasma membrane to achieve their biological functions.

protein binding and in the regulation of multiple cellular functions, like cell proliferation, differentiation and migration, angiogenesis and metastasis. Most of these processes are instrumental in the cancer progression. Typically, a majority of STs and other maturation enzymes exhibit pro- and anti-oncogenic properties depending on the cell model, the tissue and the cancer subtype, as described in this section.

NDST isoforms determine the HS N-sulfation pattern and differently influence HS fine structure and biological properties [57]. The *NDST4* gene has been suggested as a novel tumor suppressor candidate in human colorectal cancer [58] and *NDST4*-null mice strains exhibit a tissue specific phenotype with disturbed cell lineage differentiation, leading to altered colonic epithelial cell homeostasis. Knocking-down *NDST4* in mice causes a dramatic reduction of N-sulfated HS, supporting a role of this isoform in the onset of tumorigenesis and progression in colorectal cancer cells [59]. Conversely, an animal model harboring specific endothelial deletion of *NDST1* gene shows reduction of microvasculature and tumor development. This could be due to impaired interactions of N-sulfated HS with the pro-angiogenic GFs FGF-2 and Vascular Endothelial Growth Factor (VEGF) [60].

The impact of 6O-sulfation catalyzed by HS6STs in cancer-related processes has been investigated in cell lines and *in vivo* using deficient animal models. A gene expression study of the three HS6ST isoforms points out aberrant HS6ST1 and HS6ST2 overexpression during chondrosarcoma progression [61], suggesting a relationship between high HS6ST expression and tumor growth. Similarly, a significant over-expression of HS6ST2 was reported in colorectal cancer samples compared to control colonic mucosa [62], and has also been associated with tumor invasion and metastasis with a poor prognosis in gastric cancer [63]. Interestingly, gene inactivation of endogenous HS6ST2 inhibits cell migration and invasion in the pancreatic cancer cell line PANC-1 *via* Notch signaling inhibition. This effect was also confirmed by the reduced size of *in vivo* tumors following injection of HS6ST2-depleted PANC-1 cells to nude mice [64]. Altogether, this suggests a strong pro-oncogenic role of HS6ST2 isoform in digestive system cancers.

HS2ST expression was significantly upregulated in prostate carcinoma in comparison to normal tissues as the cell metastatic potential increased, pointing to the role of HS2ST in cell proliferation and invasion during prostate cancer progression. Mechanistically, silencing of HS2ST expression coincides with an accumulation of actin and E-cadherin at the tumor cell surface with a decrease of GF binding and signaling involving mainly FGFs and Transforming Growth Factor β (TGFβ) [65].

Attempts to understand how 3O-sulfation acts as a key regulator of HS function have been addressed in different pathophysiological processes, including cancer. Loss of *HS3ST2* gene expression following hypermethylation in the 5′ promoter region is observed in several types of cancer including breast, lung, colon and pancreatic cancers [66]. HS3ST2 re-expression in highly invasive MDA-MB-231 breast cancer cells results in increased cell invasion and migration compared to control cells, which is correlated to higher expression of matrix proteases and activation of Mitogen Activated Protein (MAP) kinase signaling, thus favoring an invasive phenotype [67]. More studies outlining consistent HS3ST2 silencing through epigenetic mechanisms in different cancer types will be discussed in Section 4.

HS3ST3A and 3B isoforms exhibit highly conserved ST domains and apparent similar enzyme activity towards the same modified disaccharide [GlcNS,6S-IdoA2S] [40,68]. It has been shown that increased HS3ST3B1 expression contributes to acute myeloid leukemia progression, promoting angiogenesis and tumor cell proliferation through VEGF-signaling pathway [69]. HS3ST3B1 expression was significantly upregulated in non-small cell lung cancer cells compared to control tissues. As a consequence, HS3ST3B1 has been proposed as a novel regulator of epithelial-mesenchymal transition (EMT) [70]. The tumor regulator role of HS3ST3A has been described in breast cancer, with dual activities, acting either as a pro-oncogenic or an anti-oncogenic factor in a cell- and tumor-dependent context. In the low invasive (estrogen receptor and progesterone receptor positive) MCF-7 and the invasive (triple negative) MDA-MB-231 cells, HS3ST3A transient overexpression induces cell apoptosis, whereas cell proliferation is promoted in HS3ST3A-expressing SKBR3 (Human Epidermal Growth Factor Receptor 2 (HER2) positive, HER2+) tumor cells [71]. The clinical impact of HS3ST3A expression has been investigated in a patient cohort, clearly showing that cancer progression could be specifically correlated to HS3ST3A expression in HER2+ in breast cancer patients. HS3ST3A expression can thus be considered as a discriminating biomarker for diagnosis and prognosis in this aggressive subtype of breast cancer [71].

Using an *in cellulo* model of breast cancer, the cell line MDA-MB-231, Hellec *et al.* [72] showed that independent overexpression of HS3ST2, -3A, -3B and -4 clearly leads to the same pro-tumoral activity with markedly increased cell proliferation and viability. At the molecular level, increased activation of c-Src, Akt and NF-κB signaling pathways were associated to an up-regulation of the anti-apoptotic proteins survivin and XIAP (X-linked inhibitor of apoptosis), producing HS3ST-expressing tumor cells more resistant to cell death induction [72]. Interestingly, it has been suggested that the pro-tumoral activity of the isoform HS3ST3B depends on the expression of neuropilin (NRP), a recently identified ligand of 3O-sulfated HS [20].

Recent interesting findings suggest that controlling ST subcellular localization in cells could be a new way for the cell to regulate ST activity and by extension, to influence HS cellular functions. In contrast to the HS3ST3B isoform which is localized in the Golgi apparatus, HS3ST2 was associated with syndecan-2 at an atypical subcellular localization at the plasma membrane of HeLa cells and primary macrophages [73]. The precise significance of the export of the HS3ST2 to the cell surface is unknown but may suggest that processes occurring at the cell surface require HS3ST2 enzymatic or chaperone activity. On the other hand, the role of HS and shed syndecan in the nucleus is also puzzling [73] and it would be highly interesting to determine whether their presence is associated with that of STs and in particular HS3STs. It is also intriguing to note that HS6STs are amongst the rare HS biosynthesis enzymes to be secreted in the extracellular compartment [74,75]. Although the physiological relevance of these locations is unclear, these may play additional roles in the regulation of the HS sulfation status and expand the already incredibly vast array of functions of these very special GAG chains.

4. Mechanisms of regulation of HS biosynthetic genes in cancer

As mentioned in Section 3, numerous investigations have studied the differential expression of HS synthesis and maturation enzymes in various tumors or cell lines, mainly towards the identification of potential markers of cancer onset and progression and ultimately, to provide tools for evaluating patient outcome. In the following section, differences in gene expression, resulting from genetic and epigenetic events will be discussed in different cancer types and patient cohorts.

To date, only two inherited disorders have been associated with mutations in genes involved in HSPG metabolism. The first includes mutations in the GT genes *EXT1* or *EXT2*, resulting in hereditary multiple exostoses, a rare syndrome characterized by the formation of osteochondromas or exostoses at the extremities of long bones and which, in rare cases, can degenerate into chondrosarcoma that are nasty type of sarcoma affecting bones and joints [76]. The second disorder is due to mutations in the glypican 3 (*GPC3*) gene associated with the rare Simpson-Golabi-Behmel syndrome, an X-linked disorder characterized by pre- and post-natal overgrowth with an increased risk of neoplasia, especially in young patients [77]. The identification of these two inherited conditions reinforces the idea that HS defects are associated with the tumor process.

Another aspect of gene regulation that has also been deeply investigated in the cancer field is the implication of epigenetic modifications. Epigenetics refer to heritable changes in gene expression without affecting the underlying DNA sequence [78] and predominantly include DNA methylation, histone modifications, chromatin remodeling and microRNA (miRNA) regulation. DNA methylation is one of the most studied epigenetic modifications and predominantly occurs in CpG-rich regions called CpG islands, primarily located in the promoter regions [79]. On the other hand, exploring miRNA differential expression is an emerging field in oncogenesis as miRNA, like other epigenetic elements, are deeply involved in ECM homeostasis and remodeling [80].

So far, only a few genes encoding HS-synthesizing enzymes have been reported to be epigenetically regulated and, noteworthy, most of the literature has been dedicated to genes coding HS-modifying enzymes, and to the HS3ST family in particular. Epigenetic regulation of the GT EXT1 seems to be dependent of the cancer type. Epigenetic silencing of *EXT1* due to promoter hypermethylation leads to altered HS function, thus promoting the development of acute types of leukemia and non-melanoma skin cancer [81]. Interestingly, differentially methylated regions in *EXT1* promoter were identified when comparing high-grade and low-grade prostate tumors and therefore may have a possible prognostic value [82]. In contrast, no different methylation patterns of *EXT1* gene was observed in osteochondromas and chondrosarcoma tissues or cell lines [83–85]. Finally, at the protein level, EXT1 is also considered as an early diagnosis biomarker of cholangiocarcinoma, since elevated plasma levels were observed both in an animal model of the disease and in human patients [86].

In liver tumors isolated from an animal model of spontaneous hepatocellular carcinoma, elevated percentage of CpG methylation sites concomitant with lower gene expression was observed when analyzing *EXTL1* promoter region. Epigenetic silencing was abrogated after treatment of a mouse liver cancer cell line with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, suggesting an epigenetic regulation of *EXTL1 via* DNA methylation [87]. Methylation of the promoter of the *EXTL3* gene, a homologue of *EXT1*, was reported in mucinous colorectal cancer cell lines and has been associated with decreased mRNA and HS expression in EXTL3-small interfering RNA (siRNA) transfected cells. However, *EXTL3* epigenetic regulation seems to be cell type- and context-dependent as no significant correlation between promoter methylation and patient's outcome was found [88].

NDST1 gene expression can be controlled by the level of miRNA-24 in angiogenesis leading to a lower response of endothelial cells to VEGFA *via* reduced HS sulfation [89]. In human breast cancer, the

methylation status of miRNA-149 controls the overexpression of NDST1 and modulates cancer chemoresistance associated with unfavorable patient outcomes [90]. Similarly, negative regulation of NSDT1 expression *via* miRNA-191 was associated with enhanced cell proliferation in the human gastric carcinoma cell line MGC803 [91]. Studies on the *GLCE* gene indicate that the expression of Glce can be epigenetically repressed by miRNA-218, leading to altered endothelial cell migration *in vitro* [92]. The epimerase Glce is also considered as a potential tumor suppressor gene with significant reduced gene expression in breast tumors. Mostovich *et al.* [93] reported that activation of Glce expression in breast cancer cells is due to both changes in chromatin structure and histone modifications, rather than DNA methylation. Evidence of epigenetic regulation of the HS-editing enzymes sulfatases SULF1 and SULF2 and the degrading enzyme heparanase have been reported but is beyond the subject of the present paper (reviewed in [94]).

The HS3ST2 is the most studied isoform in many cancer and tumor cell types. In 2003, Miyamoto *et al.* [66] first reported the hypermethylation status of the 5′ promoter region of *HS3ST2* associated with impaired signal transduction in several types of cancers including breast, colon, lung and pancreas. This was followed by numerous studies, all consistently reporting the hypermethylation of the *HS3ST2* gene in various malignancies such as invasive cervical [95], gastric and hematological neoplasms [96–98]. An increased methylation of *HS3ST2* promoter region in cervical dysplasia and in late-stage breast cancer cases was reported, highlighting epigenetic regulation in early but also in late stages of cancer development. *HS3ST2* hypermethylation has been also associated with poor overall patient survival in node-negative stage 1-III non-small cell lung cancer. Exogenous expression of HS3ST2 in lung cancer cell lines enables inhibition of cell migration, invasion and cell proliferation [99]. Similarly, integrative analysis of transcriptome and methylome data from patients with epithelial ovarian cancer, shows that high methylation level of *HS3ST2* gene is correlated with low RNA and protein expression as well as poor patient outcome [55]. Interestingly, the authors demonstrated that HS3ST2 was able to inhibit the malignant phenotype of ovarian cancer by compromising ligand-receptor interactions such as Interleukin 6 (IL6), FGF2 and epidermal growth factor (EGF). Altogether, the use of DNA methylation of *HS3ST2* gene stands for a valuable tool for diagnosis or prognosis purposes, with multiple possibilities and applications. However, how the epigenetic silencing of this isoform, which is barely expressed in most tissues except brain, can have such an impact on the tumor onset and patients' outcome remains a puzzling question.

In the pancreatic cancer cell line PANC1, HS3ST3B1 expression was activated by treatment with the histone deacetylase trichostatin-A and could promote EMT [64]. In 2009, we noted that an ensemble of genes coding the STs HS3ST1, HS3ST3A and HS3ST6 were hypermethylated in their promoter region and this was associated with reduced RNA expression and altered HS pattern in a chondrosarcoma cell line [85]. Interestingly, reversing the epigenetic silencing of HS3ST3A by transient expression or by treatment with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine, resulted in a reduced proliferation and migration capacity of the chondrosarcoma cells suggesting the implication of this isoform in tumor progression [85]. More recently, *HS3ST3A* gene expression was reported to be epigenetically repressed in several breast cancer cell lines but having either tumor-suppressor properties in MCF-7 or pro-oncogenic effects in (HER2+) SKBR3 cells. *In vivo* experiments in xenografted mice provide strong evidence for the HS3ST3A anti-oncogenic properties as observed in genetically engineered MCF-7-HS3ST3A cells. Finally, a high expression level of HS3ST3A in tumor tissues from HER2+ subtype patients was associated with reduced relapse-free survival as mentioned in Section 3. Altogether, this underscores the critical importance to consider the cell- and tumor-dependent context and the influence of surrounding stromal or immunity cells before systematically attributing tumor-suppressive or oncogenic activities to a gene/HS modification [71]. These data also suggest that epigenetic mechanisms affect cell-matrix interactions, as cell surface HS chains will behave and interact differently according to their structure and composition. Altogether, these studies highlight the importance of epigenetic regulation in modulating gene expression, and by consequence the HS profile and functions. Because these mechanisms are known to be reversible and could therefore be used to slow cancer progression, they could also be exploited for diagnosis purposes and for informing on cancer stage and/or severity, and for therapeutic purposes.

Alterations in HS fine structure can result from transcriptional regulation of enzymes involved in HS elongation and maturation. Although up- or down-regulations of genes involved in the biosynthesis or maturation of HSPGs have been reported in several types of cancers, transcript levels considerably varied, depending on enzyme isoforms, nature of tumor tissues and metastatic tumor status or progression. Transcriptomic approaches based on microarray technology have reported differential expression of several enzymes in order to provide a better view and understanding on the cancer development process. In 2013, Fernández-Vega *et al.* found a differential expression of HS3ST transcripts in infiltrating ductal adenocarcinoma (IDA) depending on the presence or absence of metastasis [99]. The expression of the *HS3ST6* gene was significantly downregulated in both types of IDA while the expression of *HS3ST4* was even more reduced in the metastatic form [100]. More recently, the same group identified differential expression of enzymes involved in the biosynthesis and maturation of HS in right-sided colorectal cancer (CRC). However, greater differences were observed in metastatic CRC [101]. Significant changes in NDST, HS6ST and HS3ST transcript levels have been described in left-sided CRC, depending on the presence or absence of metastases [102]. In the same context, significantly reduced expression of *EXT2*, *HS6ST1* and *HS6ST2* genes is a hallmark of higher tumor grade of human glioma cells [103]. HS length and sulfation pattern acts as interdependent mechanisms as has been observed in breast carcinoma cells with differences between EXT expression and HS fine structure [104]. This series of studies indicates a relationship between HS alteration (in terms of elongation and sulfation) and the metastatic properties of the tumor. Numerous reports have provided evidence for the relationship between gene expression and the corresponding HS modification. However, more efforts are clearly needed, as gene expression cannot predict exactly how the HS chain will be finely affected.

5. Typical examples of ligands binding to sulfated HS motives with a focus on 3O-sulfated HS and their relevance in cancer

Although a very large number of HS ligands have been described, few of them have been reported to bind 3O-sulfated motifs [105]. A typical example 3O-sulfated HS ligand is the anticoagulant AT-III. The biological activity of AT-III is due to its ability to bind a specific HS pentasaccharide [GlcN(NS,6S)-GlcA-GlcN(NS,3S,6S)-IdoA(2S)-GlcN(NS,6S] (Table 1). This structure contains a 3O-sulfate group, which is transferred onto a N-sulfated and 6O-sulfated glucosamine by the HS3ST1 and HST3ST5 isoforms [46,106,107]. Interestingly, an additional 3O-sulfated group on a glucosamine residue of longer oligosaccharides, such as an octasaccharide, increases AT-III affinity for HS in comparison to the mono-3O-sulfated pentasaccharide [106]. This is a typical example illustrating the importance of HS 3O-sulfation for biological activity of HS and pointing to the crucial role of HS3STs in creating biologically active molecules. The viral envelope gD of HSV-1 also specifically binds to 3O-sulfated HS chains which are used as an entry receptor for the virus and contributes to cell-cell fusion before infection [108,109] (Table 1). The four isoforms HS3ST2 [49], HS3ST3 [110], HS3ST4 [111] and HS3ST5 [47] have been shown to catalyze the sulfate transfer onto the position 3 of N- and 6O-sulfated glucosamine, creating HSV-1 gD binding site on HS, and resulting in 3O-sulfated oligosaccharides that have presumably other functions besides conferring susceptibility to viral infection.

Table 1
Main HS 3O-sulfated binding proteins and their implication in cancer.

Target proteins	Associated biological functions	*in vitro*, *in cellulo* and *in vivo* models	Conducted studies	References
Antithrombin-III (AT-III)	Inhibition of coagulation cascade	Synthetic HS HS fragments from enzymatic degradation of heparin	Structural and biochemical characterization of AT-III HS binding pentasaccharide sequence	Guerrini et al., 2013 Hricovíni et al., 2001 Shriver et al., 2000
Herpes Simplex Virus (HSV) glycoprotein D (gD)	Cell infection	Human mesenchymal stem cells (hMSC) and HeLa cells for *Herpes Simplex Virus* entry assays, hMSC expressing HSV-gD for interaction studies	Study of the role of 3O-sulfated HS as entry receptors for HSV-1 in target cells	Choudhary et al., 2011
		Primary human corneal fibroblasts	Study of the role of 3O-sulfated HS in HSV infection	Tiwari et al., 2005
Growth factors				
FGF7	Activation of FGFR2IIIb	FGF7 crystals/Hep fractions	Structural studies of FGF7/HS binding domain Protease protection assays	Ye et al., 2001
FGF7	Cell proliferation	Breast cancer cell lines	Pro- or anti-proliferative effects and signaling studies	Mao at al., 2016
FGF10	Cell expansion	Progenitor cells	Progenitor expansion during organogenesis & development	Patel et al., 2014
TGFβ	Epithelial-to-mesenchymal transition (EMT)	Non-small cell lung cancer cell lines	Induction of the EMT by HS3STB1	Zhang et al., 2018
Morphogens				
Wingless (Wnt)	Morphogenesis, development Tumor growth	Synthetic HS HEK293 cell line expressing a Wnt reporter gene	Wnt/HS oligosaccharides interaction studies Test of a high-affinity human monoclonal antibody (HS20) targeting HS chains of GPC3 and Wnt / Immunotherapy	Gao et al., 2016
Chemokines				
Cyclophilin (CypB)	Migration and integrin-mediated adhesion of T lymphocytes	Peripheral blood T lymphocytes and Jurkat T cell line, CD4+ lymphocytes, monocytes/macrophages and related cell lines	HS structural and functional studies of CyPB/HS binding site and HS3ST3 expression analysis	Vanpouille et al., 2007 ; Deligny et al., 2010
Glycoproteins/Receptors				
Neuropilin-1 (NRP1)	Angiogenesis and axon guidance Tumor progression and cell viability	Bovine, mouse and human sera, mouse embryo dorsal root ganglion and neurons, Human umbilical vein endothelial cells (HUVEC) Breast cancer cells	Axonal growth modulation and endothelial cell sprouting, binding assays Impact of the modulation of NRP-1 and HS3ST expression in cancer cells	Thacker et al., 2016 Hellec et al., 2018
Natural Killer cell receptor, Killer cell Ig-like Receptor (KIR) 2DL4	Immune system activation, cytokine production and cytotoxicity/anti tumor effect	Natural Killer cells, HEK293T cells expressing recombinant KIRD2DL4	Regulation of cytokine production by KIR2DL4-expressing NK cells Receptor endocytosis and membrane trafficking KIRD2DL4-HSPG interaction	Brusilovsky et al., 2013 ; Brusilovsky et al., 2014

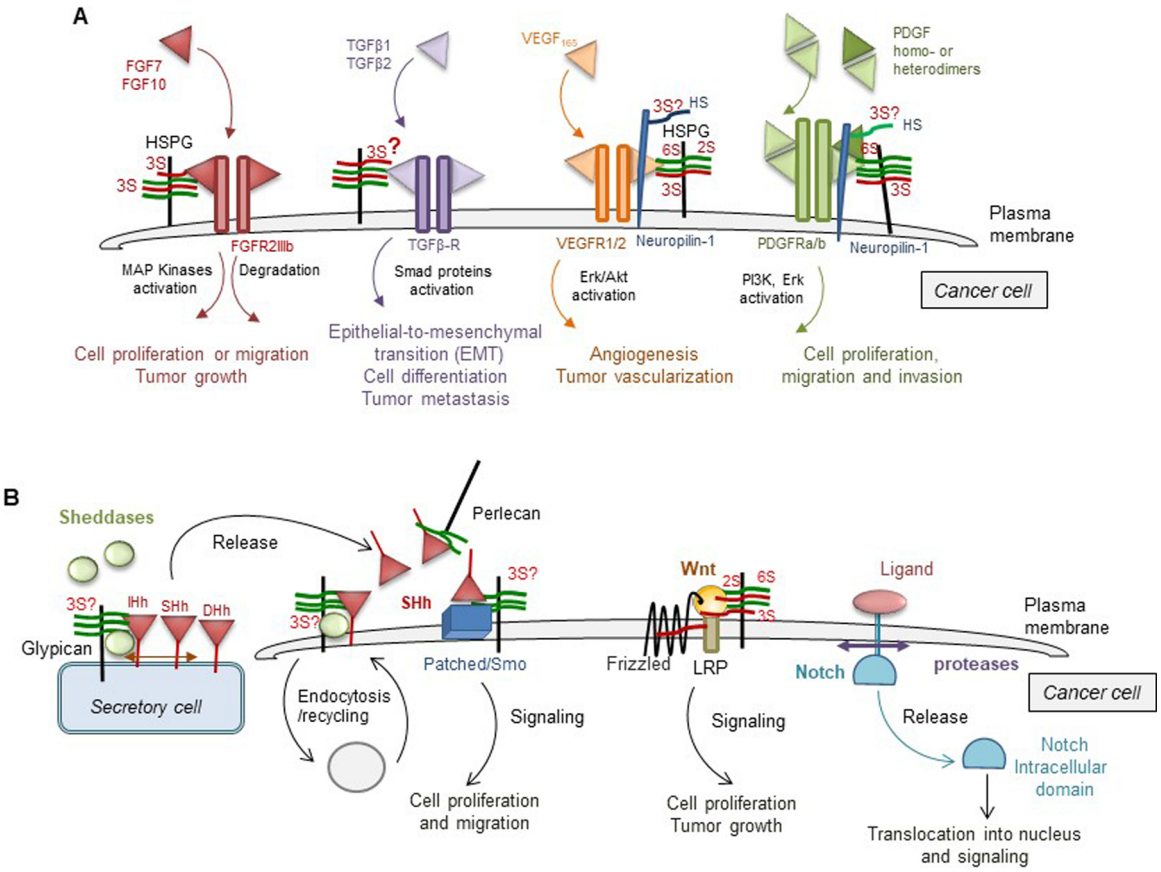

Fig. 2. Interactions of growth factors (GFs) and morphogens with sulfated heparan sulfate (HS) motives and their pathophysiological functions in cancer. The terminal and rare 3O-sulfation, catalyzed by HS3STs, produces 3O-sulfated motives which constitute specific and high affinity binding sites for a series of ligands, controlling their biological activity in cancer cells. 3O-sulfated HS chains are colored in red. **A. GFs-HS interactions**. Fibroblast Growth Factor 7 (FGF7) and Fibroblast Growth Factor 10 (FGF10) are responsible for cell proliferation and migration through FGFR2IIIb receptor activation or degradation. Transforming Growth Factor β (TGFβ) induces endothelial-mesenchymal transition (EMT) and cell differentiation, mainly *via* Smad protein activation. Biological activity of the pro-angiogenic factor Vascular Endothelial Growth Factor (VEGF) can be regulated by another proteoglycan, neuropilin-1 (NRP1) to promote angiogenesis and tumor neovascularization. Platelet-derived growth factor (PDGF) is a mitogenic GF which induces cell proliferation and migration in cancer cells in association with NRP1, acting as a co-receptor. **B. Morphogens (Hedgehog (Hh), Wingless (Wnt) and Notch)-HS interactions.** Hedgehog (Hh) signaling contributes to embryonic development *via* the action of three identified mammalian homologues: Sonic Hedgehog (SHh), Indian Hedgehog (IHh) and Desert Hedgehog (DHh). HS chains can potentially act as scaffolds for Hh ligands and sheddases, which is important for their turnover and depend on HS sulfation degree and promote Hh-Patched/Smo-mediated signaling. Wingless (Wnt) factor binds to Frizzled receptor and to different co-receptors, as Lipoprotein Receptor-related Protein (LRP), to promote cell signaling and tumor cell proliferation through sulfated HS chains interactions. The transcription factor Notch is released from plasma membrane and migrates to nucleus after ligand binding. Notch signaling is involved in many pathophysiological processes, like embryonic development, human developmental disorders and several types of cancers.

5.1. Role of HS in cell proliferation and tumor growth – Interaction of FGF and TGFβ with sulfated HS motives

The demonstration that HS chains are essential for the formation of a high-affinity FGF-Fibroblast Growth Factors Receptor (FGFR)-HS ternary complex is one of the first identified and still most important examples of the role played by HS in the regulation of soluble mediators' biological activity [112,113]. This paragraph will discuss the role of HS chains as regulators of GF biological activity, in particular as co-receptors and will underscore the impact of 3O-sulfation.

Eighteen mammalian FGFs (FGF1–FGF10 and FGF16–FGF23) have been identified and classified into six subfamilies, based on sequence homology and phylogenetic evolution [112]. They specifically interact with their cognate tyrosine kinase receptors (FRFR1-4) and with HS chains to regulate multiple cell signaling activities (Fig. 2A). The FGF-FGFR binding specificity and affinity are determined by differences in the primary sequences among FGFs and FGFRs and in their spatio-temporal expression patterns; they also depend on specific HS sulfation motifs [113]. The structure of the ternary FGF-FGFR-HS complex and its stoichiometry (2:2:2 or 2:2:1) and how FGF and HS cooperate to dimerize the receptor are still debated and have been discussed elsewhere [114,115]. FGF-HS interactions play a key role in embryonic development, regulating major cellular processes including cell proliferation, differentiation, survival and migration, which makes FGF signaling susceptible to subversion by cancer cells [116]. Aberrant FGF-HS interactions in the tumor pathogenesis mainly depend on HS sulfation abundance and profile. In this regard, the influence of 2O- and/or 6O-sulfation on FGF1 and FGF2 binding in cell proliferation has been mainly studied. 2O- and 6O-sulfation of HS is clearly important in FGF1 binding and in mediating its mitogenic activity [117,118]. An [IdoA2S-GlcNS,6S-IdoA2S] trisaccharide motif is implicated in high-affinity FGF1 binding [119] and crystallographic studies reveal a direct role of 2O- and 6O-sulfate groups in HS-FGF1 interactions [120]. Some reports indicated that both 2O- and 6O-sulfations are also important for the mitogenic activity of FGF2 [118], whereas others questioned the requirement of 6O-sulfates for such activity [117]. Indeed, binding and crystallographic studies did not conclusively showed an essential role of 6O-sulfation in the binding of HS to FGF2 [121]. Alternatively, FGF2 is thought to interact with FGFR to form a FGF-FGFR-HS ternary complex able to trigger cell signaling [122]. In mouse and human tumors, highly

6O-sulfated HS induced the formation of ternary HS-FGF1 or FGF2-FGFR1 complexes and promoted angiogenesis and metastasis [123]. FGF1-induced proliferation was also dependent on 2O- and 6O-sulfated HS motifs in human retinoblastoma cell lines [124]. FGF2 ability to stimulate tumor cell growth has been described (i) in glioma cell lines, where the mitogenic signal was correlated with the abundance of 2O- and 6O-sulfated HS disaccharides [125]; (ii) in MCF-7 breast cancer cells [126] in which FGF binding kinetics have been related to their ability to stimulate tumor cell growth [127]; and (iii) in endothelial cells, in which it was shown that FGF2 mitogenic activity requires specific 6O-sulfated HS domains to enhance FGF2 growth-promoting activity [128].

Beside 2O- and 6O-sulfation, early indirect studies have suggested a role for HS 3O-sulfation in the control of FGF binding to their receptors. It was shown that the anti-coagulant AT-III binding motif of Hep (that contains a GlcNS-3S(± 6S)) interacts with the ectodomain of FGFR to form a binary complex competent for FGF binding and signaling [129]. However, the importance of HS 3O-sulfation in FGF binding, signaling and cancer is less documented, possibly because it is a technically challenging modification to investigate compared to 2O- and 6O-sulfation. In general, this modification is not considered to play an important part in the interactions between HS and FGF1 or FGF2 and their receptors. However, recent binding and crystallographic studies revealed that the 3O-sulfate group of a synthetic HS disaccharide [GlcNS,3S-IdoA2S] forms a ionic interaction with lysine residues of FGF1 [130] and FGF2 [131] that enhances its binding affinity, supporting the assumption that 3O-sulfation may be more critical in fine-tuning the affinity of FGF to their receptors, than generally considered.

In addition, there is increasing evidence for a role of 3O-sulfation in regulating FGF7 and FGF10 signaling and activities. FGF7 and FGF10 belong to the FGF7 subfamily, one of the five paracrine-acting FGF subfamilies that use HS as mandatory co-receptors [132,133]. HS promote FGF7 members signaling by orchestrating the formation of a symmetric 2:2 FGF-FGFR dimer, thus contributing to a directional paracrine signaling system from the stromal to epithelial compartment. Intercompartmental homeostasis supported by FGF7 or FGF10-FGFR2IIIb-HS complex formation is mandatory in embryonic development and is compromised in many epithelial solid tumors. FGF10 has been implicated in prostate [134] and pancreatic cancer [135], and FGF7 plays an important role in many cancer types. Although the role of HS as co-receptor of FGFR2IIIb is well-established, the contribution of HS chains in the tumorigenesis process is rarely considered, but it is likely to be significant [136]. Structural studies reveal that FGF7 possesses a unique Hep binding domain that, in contrast to FGF1 and FGF2, interacts only with Hep oligosaccharides with anti-coagulant activity [137,138]. In line with this, in protease protection experiments, FGF7 was specifically protected by Hep oligosaccharides when being of sufficient length and containing a 3O-sulfate group [17]. Thus, it appears that the strict requirement for 3O-sulfation for FGF7 and FGF10 binding to HS is the most stringent criteria for an interaction between HS and any GF studied to date, and this has potentially major biological implications. Several studies point to a role of 3O-sulfation in governing different binding of FGF7 and FGF10 to FGFRIII2b, and downstream inducing different cell behavior. Indeed, FGF7-HS stimulation leads to FGFRIII2b ubiquitin-driven degradation and, ultimately, to cell proliferation. Alternatively, FGF10-HS triggers additional phosphorylation of FGFR2IIIb, leading to the recruitment of PI3K and SH3 binding protein 4 (SH3BP4) and promoting endosomal receptor recycling and cell migration [139,140]. An intriguing possibility for explaining the different FGF7 *versus* FGF10-induced pattern of FGFR2IIIb tyrosine phosphorylation and cell response could be that the two ligands recruit specific HS motifs, possibly differing by the arrangement of 3O-sulfates that induce different affinities of the GFs for their cognate receptor. Supporting this concept, the alteration of 3O-sulfate epitope by differential regulation of HS3STs modulates the response to FGFRIII2b signaling and control cell progenitor expansion during organogenesis [18].

Altogether, HS appear as major components of the FGF-signaling axis for both endocrine and paracrine FGFs. 3O-sulfation adds an additional note of specificity and sophistication to the system since it is capable to finely regulate the biological functions of FGFRs, in particular to switch the activity of FGFR2IIIb from homeostasis to proliferation. Detailed mechanisms of this exquisite regulation await further investigation.

Transforming growth factor β (TGFβ) is a potent regulator of tumor metastasis. This GF induces EMT in endothelial and epithelial cancer cells, mainly by activating the Smad signaling pathway, leading to TGFβ-induced cell differentiation and proliferation [141,142] (Fig. 2A). Among the 40 cytokines of the TGFβ superfamily, around a third, including TGFβ1, TGFβ2 and various Bone Morphogenic Proteins (BMP), are currently known to bind to Hep and HS with a specificity depending on the TGFβ isoforms and Hep/HS structures [143,144]. The capacity of TGFβ1 to interact with HS was first established by McCaffrey *et al.* [145]. Subsequently, Lyon *et al.* [143] showed that Hep and highly sulfated liver HS bind both human TGFβ1 and TGFβ2, but not TGFβ3, and proposed a structural model for this interaction. In pathological situations, it has been shown that TGFβ stimulates HSPG expression in human colon carcinoma cells, mainly due to higher core protein mRNA expression level [146]. In the same context, increased HS expression at the tumor cell surface contributes to enhanced matrix production, promoting cell proliferation through GF signaling regulation. HSPG syndecan-2 down-regulation inhibits TGFβ-induced Smad activation in fibrosarcoma cells, which alters tumor cell adhesion [147].

Batool *et al.* [148] showed that the reduced level of 6O-sulfation of HS in breast cancer MCF7 cells and in the human epithelial cell line FaDu (from a squamous cell carcinoma of the hypopharynx) affects the ability of TGFβ1 to signal *via* its receptor and to elicit a growth response. With regards to HS modifying enzymes, HS6ST2 has been identified as a critical factor for TGFβ-induced IL11 production in a breast cancer bone metastasis *in vivo* model. Reduction of tumor growth and bone lesions have been observed in a mouse model, using a high molecular weight *E. coli* K5-derived sulfated Hep-like polysaccharide. These results demonstrate a critical role of sulfated HS/Hep oligosaccharides in cancer cell progression and invasion, and the potential anti-metastatic effect of sulfated Hep-like oligosaccharides through Smad pathway inhibition [149].

With regards to 3O-sulfation, it has been recently reported that HS3ST3B1 mRNA was up- regulated by TGFβ in non-small cell lung tumors and in mesenchymal cell lines, suggesting a possible role of 3O-sulfated HS (produced by HS3ST3B1) in TGFβ-mediated EMT [70]. Although TGFβ was the first HS-binding protein to be discovered, molecular information regarding HS function in TGFβ activity is more limited compared to the FGF family. Interestingly, HS could be implicated in TGFβ pluripotency as, in contrast to most other cytokines, TGFβ acts as a bi-functional regulator that has both stimulatory and inhibitory activity in the same cells. A major complication in attempting to assess the role of HS in TGFβ and BMP signaling within the tissues is the presence of various agonist and antagonist proteins. Interestingly, a high proportion of these proteins could bind Hep and/or HS with high affinity but add complexity to these regulation processes. Overall, investigations in this area remain limited.

5.2. Role of HS in angiogenesis and tumor vascularization: Interaction of VEGF and PDGF with sulfated HS motives

Physiological wound healing and tumor angiogenesis are modulated by the sequential and carefully orchestrated release of growth stimulators and inhibitors. These regulatory small molecules are produced by the activated endothelium. They selectively interact with sulfated HS attached to shed or membrane-bound HSPGs, depending on the HS sulfation profile [60,150]. New vessel formation and wall cell recruitment are modulated by GF interaction with HS, including Platelet-Derived Growth Factor (PDGF) and VEGF. Thus, HS chains play an

important part in the control of tumor angiogenesis on both endothelial and cancer cells, suggesting a potential use of HS as targets for novel anti-angiogenic therapies [151].

VEGF is a potent pro-angiogenic GF that is a crucial actor in tumor angiogenesis. $VEGF_{165}$ is the most abundantly expressed splice variant and it interacts with HSPGs and NRP to bind two receptors, VEGFR1 and VEGFR2, which mediate downstream signaling activities (Fig. 2A). Highly sulfated HS domains containing 2O-, 6O-, and N-sulfates significantly contribute to the binding of HS to the $VEGF_{165}$ homodimer [152]. 6O-sulfation is the most essential modification in this regard [153]. The use of Hep oligosaccharides indicated that the 3O-sulfate group does not play a major part in $VEGF_{165}$ binding *in vitro* [154]. In endothelial cells, the ability of HS fragments to interfere with $VEGF_{165}$ interactions increases with oligosaccharide length and N- and 2O-sulfation levels [155]. This suggests a model where $VEGF_{165}$ facilitates the association of Hep/HS with VEGFR-2 to generate a high-affinity ternary complex between $VEGF_{165}$-VEGFR2-HS. Since NRP1 synergizes with VEGFR2-$VEGF_{165}$ complexes to enhance Hep binding, a quaternary high affinity complex including all components was even proposed [153]. Nevertheless, the detailed structural features governing the formation of these complexes are not yet established and the potential implication of 3O-sulfates in VEGF-HS-NRP-VEGFR complex formation is unknown. However, since recent findings support the role of 3O-sulfated epitopes in HS binding to NRP1, as described in more detail below, they may play an active role in modulating angiogenesis [19].

In the context of cancer, 2O-, N-, 6O- and N-, 6O-, 3O-sulfate levels are increased in the endothelium of ovarian tumors [128]. Interactions of sulfated HS oligosaccharides with $VEGF_{165}$ inhibits VEGF receptor activation and signaling and reduce GF-mediated endothelial cell migration and proliferation [155]. Interestingly, endothelial cell functions, in particular angiogenesis, are disrupted when 6O-sulfation is impaired following *HS6ST1* or *HS6ST2* silencing with short hairpin RNA (shRNA) [128]. Cell growth and vascularization of subcutaneous lung carcinoma were also decreased in *NDST1*-deficient mice. The study of GF distribution in tumor sections showed reduced VEGF binding to tumor endothelium and modified GF signaling when HS N-sulfation is altered [60]. Although 3O-sulfation has not yet been shown to assume a prominent part in VEGF and VEGFR binding, its role may have been underestimated [55]. Indeed, it has been shown that HS3ST3B1 positively contributes to acute myeloid leukemia progression *in vitro* and *in vivo* by inducing VEGF expression and shedding. The effects of HS3ST3B1 on the activation of Erk and Akt can also be blocked by the VEGFR inhibitor axitinib, which is suggestive of a relationship between HS 3O-sulfation and VEGF-activated signaling pathways [69]. Interestingly, by an unbiased approach, Thacker *et al.* [18] identified six new 3O-sulfate binding proteins, including in first instance NRP1, as well as amyloidβA4, biglycan, clusterin, hyaluronan binding protein 2 (HABP2) and mannose-binding protein C. The importance of 3O-sulfation in the functions of NRP1 has been validated in binding assays and *in cellulo*. Of note, 3O-sulfated HS were potent inhibitors of VEGF-mediated endothelial spouting, in accord with a potential impact on tumor angiogenesis.

NRP1 is a multifunctional transmembrane co-receptor protein playing a major role in several developmental processes, such as vasculogenesis, and in immunoregulation. Accumulating evidence has associated NRP1 expression with tumor progression *via* its interaction with a large number of mediators as semaphorins, VEGF, EGF, Hepatocyte Growth Factor (HGF), PDGF and their cognate signaling receptors [156] (Fig. 2A). Supporting a role of HS 3O-sulfation in the cancer process *via* NRP1, Hellec *et al.* [20], recently showed by NRP1-knockdown experiments in MDA-MB-231 cells, that the tumor-promoting effects of HS3ST3B is a NRP1-dependent mechanism. Of note, NRP1 is itself a PG that can be modified with either HS or chondroitin-sulfate chain on a single serine residue. Differential GAG modification of NRP1 regulates VEGF signaling in vascular endothelial cells and smooth muscle cells [157]. However, it has not been investigated whether this process depends upon 3O-sulfation. Altogether, these recent studies indicate that 3O-sulfation driven by HS3STs is an important factor in the generation of biologically active motifs for NRP1. Taking into account the pleiotropic effects of NRP1 in cancer cells, this may be of wide biological significance.

Platelet-derived growth factor (PDGF) is a mitogenic GF that is able to mediate proliferating and migrating roles in developing and cancer cells. PDGFs are dimeric molecules of disulfide-bonded polypeptides that occur as five homo- and heterodimer forms (PDGF-AA, -BB, -AB, -CC and -DD) and organized from four different polypeptide chains, that signal *via* two structurally related tyrosine kinase receptors, PDGFRa and PDGFRb [158] (Fig. 2A, Table 1). *In vitro* binding of HS-derived oligosaccharides to PDGF-BB has been shown to be dependent on the overall HS sulfation, without requirement for specific sulfated sequence [159]. In mouse models defective for N-sulfation, retention of PDGF-BB at cell surface was affected, resulting in a disruption of pericyte recruitment, showing the importance of HS sulfation in PDGF-BB-HS interactions during vascular development. Reduced N-sulfation also impaired PDGF-BB signaling and cell migration, but not proliferation [159]. In Caco-2 human colon carcinoma cells, disaccharide analysis revealed higher 2O-sulfated iduronic acid content and reduced 6O-sulfated groups in N-sulfated regions, in differentiating cells (phenotypically similar to enterocytes) compared to undifferentiated cells. The structural changes were found to affect PDGF-AA-HS interaction [160]. In mouse mammary carcinoma cell lines, a major downregulation of 6O-sulfation of glucosamine units was observed with no difference in N- and 2O-sulfation rate following malignant cell transformation [161]. Altogether, these studies underscore the contribution of HS-sulfation in vascular development and neovascularization. To our knowledge, 3O-sulfation has not been reported to be specifically implicated in HS binding to PDGF during the cancer process. However, since NRP1, as for VEGF, is an important co-receptor for PDGF signaling [162], and since 3O-sulfates are key to HS-NRP1 interactions, it may be anticipated that 3O-sulfation would interfere with PDGF signaling.

5.3. Role of HS in embryonic development and in cancer: Interaction of Hedgehog (Hh) and Wingless (Wnt) morphogens with sulfated HS motives

Morphogens, including Hedgehog (Hh), Wingless (Wnt) and Notch factors, are signaling proteins that dictate cell fate and tissue development during embryogenesis, and represent another important category of HS binding proteins with many implications in cancer [163]. The Hedgehog (Hh) signaling pathway uses three homologues identified in mammals: Sonic Hedgehog (SHh), Indian Hedgehog (IHh) and Desert Hedgehog (DHh). The cholesterol moiety anchors the Hh proteins to the cell membrane by interacting with cell surface-HSPGs, a characteristic feature that is critical for Hh transport and signaling [164] (Fig. 2B). SHh plays an important role in cell proliferation and cancer progression. It stimulates rhabdomyosarcoma cell proliferation by interacting with highly sulfated glypican-5 [165] and promotes aggressive and metastatic prostate cancer cell growth due to formation of more complexes with perlecan [166]. Glypican-5 containing 2O-sulfated IdoA HS chains acts as SHh co-receptor to promote cell proliferation [167]. In that context, HS chains are likely to be involved in SHh multimerization and cell membrane association [168]. In addition, HSPGs regulate SHh release and processing at cell surface, playing an activator role for Hh ligands in pancreatic cancer cells [169]. It was suggested that HS may act as a scaffold for SHh ligands and sheddases required for their turnover. The release of Shh would depend on the degree of HS sulfation. No direct involvement of 3O-sulfation has been reported in Hh-HS interaction in a tumor context. In this regard, the membrane-bound HSPG glypican-3 binds both Hh and Wnt (Wingless, Wg) factors through sulfated HS chains and promotes tumor cell proliferation in hepatocellular carcinoma, one of the major forms of primary liver cancers [170]. In the invasive MDA-MB-231 breast cancer cells, an upregulation of the transcription factor Tcf4, an important down-

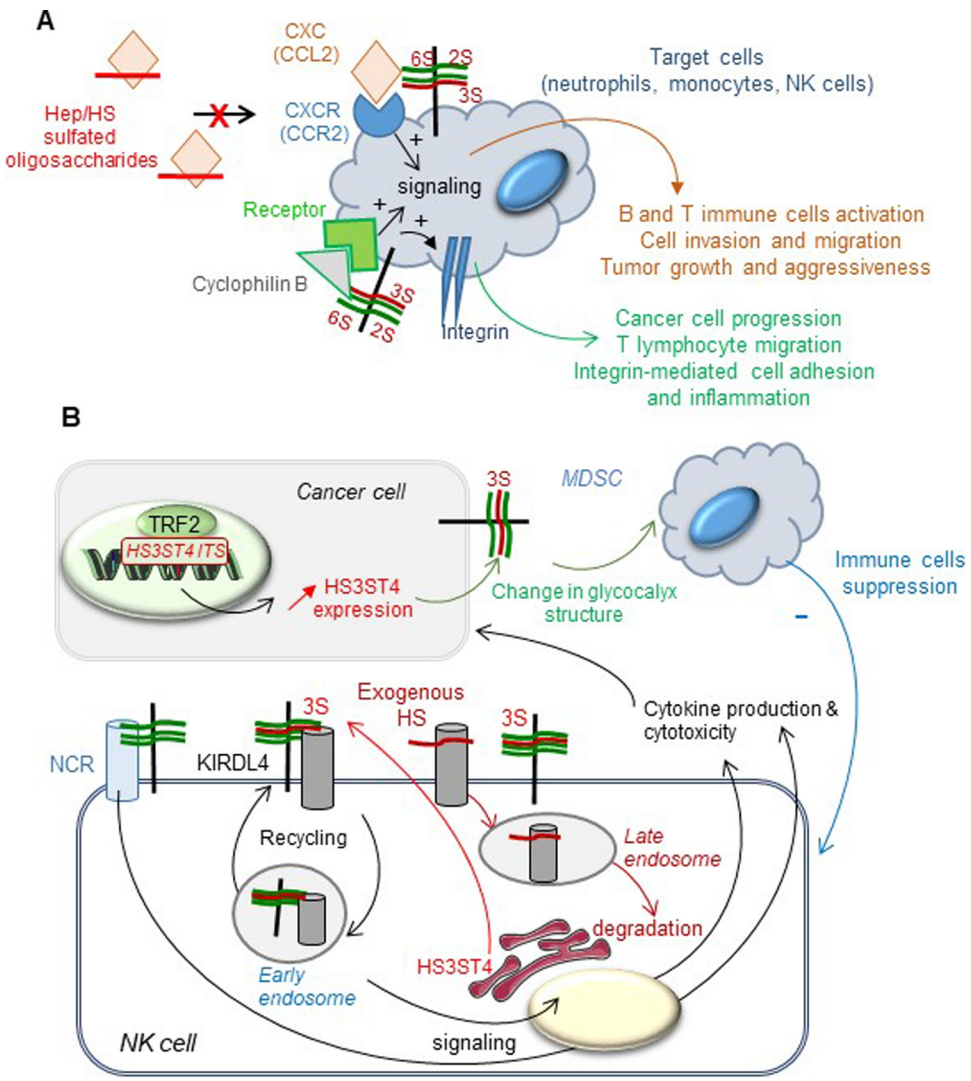

Fig. 3. Roles of sulfated heparan sulfate (HS) and their ligands in inflammation and immunity. **A. Interactions of sulfated HS with chemokines and cytokines**. Activation of CXC receptors (CCXR) by CXC chemokines is mediated by HS, leading to tumor cell proliferation, migration and invasion. N-, 2O-, 6O- and 3O-sulfated Hep/HS analogues can interact with CXC chemokines, preventing their fixation to their receptor and inhibiting CXCR activation. Cyclophilin B is involved in integrin-mediated cell adhesion and inflammatory signaling, contributing to immune cell activation and tumor proliferation. **B. Interactions of sulfated HS with immunity receptors.** 3O-sulfation, mediated by HS3STs, controls Natural Killer (NK) cells recruitment and cytotoxicity *via* interaction with NK cell activating receptors including activating forms of Killer cell Ig-like Receptor (KIR) and Natural Cytotoxicity Receptors (NCR). The activation of these receptors implicating HSPGs stimulates cell cytotoxicity and cytokine production, leading to inhibition of tumor growth. Telomeric Repeat-Binding Factor 2 (TRF2), a subunit of the shelterin complex, binds an interstitial telomeric sequence (ITS) of *HS3ST4* gene and induces its expression and HSPG biosynthesis in tumor cells, modifying the tumor microenvironment. This leads to the activation of Myeloid-Derived Suppressor Cells (MDSCs) that inhibits the recruitment of NK cells and leads to immune response escape and tumor progression.

stream effector of the Wnt/βcathenin signaling pathway, has been described when HS3ST2 is expressed in tumor cells, thereby activating the transcription of target genes involved in cell proliferation and survival [67]. HS interactions with Wnt require 2O- and 6O-sulfations. A higher binding affinity was noted when additional 3O-sulfation was present within HS-Wnt oligosaccharide binding site, underscoring the part played by 3O-sulfation in HS-Wnt interaction and signaling [171]. Targeted immunotherapy using anti-glypican-3 monoclonal antibodies has shown anti-proliferative activity in subcutaneous ectopic xenografts of human liver cancer cell line HepG2 in mice [170]. The same approach has been proposed in hepatocellular carcinoma cells by developing a human monoclonal antibody targeting the HS chains of glypican-3, leading to inhibition of the Wnt/βcatenin pathway with promising anti-tumor activity in mice xenograft models [172]. The ability to block Wnt-HS interaction with Wnt inhibitory antibodies or synthetic HS oligosaccharides with different degrees of polymerization and sulfation was studied and, importantly, showed that the antibodies bound more strongly and inhibited Wnt signaling more efficiently when 3O-sulfation was also present on N- and 6O-sulfated glucosamine residues [171]. These studies provide evidence for the importance of the nature and the number of HS sulfate groups in morphogen interactions and their associated biological effects in cancer. They clearly emphasize that targeting 3O-sulfation should be taken into account and better exploited for therapeutic development targeting these pathways, in particular in immunotherapy.

Notch is a plasma membrane associated transcription factor that is released and migrates to nucleus after ligand binding. After cleavage by proteases, the free Notch intracellular domain is translocated into the nucleus, where it targets DNA-bound proteins to activate transcription of selected target genes. Notch signaling is involved in many biological processes, like embryonic development and homeostasis [173] (Fig. 2B, Table 1). Altered Notch signaling (up- and down-regulation) has been described in human developmental disorders and cancers as uterine cervix carcinoma [174], head and neck squamous cell carcinoma [175] and hepatocellular carcinoma [176]. A positive correlation between the HSPG syndecan-1 and Notch-1 and -3 expression has been demonstrated in triple negative inflammatory breast cancer cells, highlighting the important modulator role of syndecan-1 in Notch signaling. Up-regulation of these factors is associated with inflammation and tumor progression, leading to the proposal of syndecan-1 as a potential new molecular marker in inflammatory breast cancer patients with prognostic and predictive values [177]. Focusing on O-sulfation events, Kamimura *et al.* [178] showed that *Drosophila* HS3ST-B could influence Notch signaling, reducing cell surface associated Notch protein following a loss of *Hs3st-B* expression in cells, leading to pathological neurogenic phenotypes in this animal model. In the context of cancer, human HS6ST2 potentiates Notch signaling in pancreatic cancer cells, leading to tumor growth and tumorigenicity through Notch-mediated EMT and angiogenesis [64]. No direct implication of HS 3O-sulfation in Notch signaling modulation has been reported to date in cancer.

5.4. Role of HS in cancer, immunity and inflammation: Interactions with chemokines, cytokines and immune cell receptors

It is increasingly recognized that HSPGs make a significant contribution to inflammation and immune response [179–181]. They can regulate activation and proliferation of B- and T-cells [182,183] and control leukocyte recruitment in inflammatory processes [184], mainly through interactions of HS chains with cytokines and/or chemokines [185]. HS chains also contribute to immune surveillance in cancer and have been investigated in normal and tumor cell cultures [186] or in tumor-bearing mice, showing modulation of immune cell proliferation and induction of cytokine production by cytotoxic lymphocytes *in vivo* [187]. They represent highly promising targets in immuno- and anti-tumoral therapies [188,189].

The CXC family of chemokines and their receptors (CXCR) are implicated in tumor inflammation and immunity, which are fundamental components of the cancer process [190,191]. Activation of CCXR by CXC chemokines is mediated by HS [192–194] (Fig. 3A) and has been mainly associated with tumor aggressiveness in human lung cancer cells [195], and with migration and cell invasion in human hepatoma cells [196] and in human carcinoma HeLa cells [197] (Table 1). The potential prognosis value of this chemokine family has been studied in several types of cancers, such as colorectal cancer, since CXCR activation pathways have been correlated to tumor aggressiveness and poor prognosis [198]. Targeting tumor-promoting chemokines could be a way to stop tumor growth and prevent metastasis formation, as it has been investigated for CCL2 chemokine in breast cancer cells [199]. The impact of sulfation on CCL2-HS interactions has been studied *in vitro* using N-, 2O-, 6O- and 3O-sulfated Hep analogues in the presence of recombinant CCL2 chemokine. 2O- and N-sulfated oligosaccharides display stronger binding affinity than 6O-sulfated analogues [200], but 3O-sulfation is not crucial for CCL2-GAG binding [201]. CXCL14 can stimulate NCI-H460 human lung cancer cell proliferation and migration by interacting with sialic acid and HSPG, thereby triggering NF-kB signaling and eliciting a pro-oncogenic effect [195]. A HS-derived dodecasaccharide containing 6O-sulfated glucosamine displayed interesting inhibitor chemokine- and sulfation-dependent functional effects in an endothelial cell monolayer wound healing assay and leukocyte transmigration mediated by CXCL8 and/or CXCL12 *in vitro* [202].

Another HS binding protein implicated in inflammation is Cyclophilin B (CyPB), a cytokine from the cyclophilin family, involved in integrin-mediated cell-to-cell communication and inflammatory signaling (Fig. 3A). CyPB has been associated with malignancy and tumor progression in breast cancer [203] and gastric cancer [204,205], and it has been proposed as a potential early diagnostic candidate biomarker in pancreatic cancer [206]. Its role in cancer cell protection against hypoxia and in cisplatin-induced apoptosis resistance has been described in human hepatocellular carcinoma [207] and in colorectal cancer, in which chemoresistance has been linked to higher p53 degradation following CyPB overexpression [208]. CyPB interacts with cell surface O-sulfated HS, triggering peripheral blood T lymphocyte migration and integrin-mediated adhesion [209]. Determination of the structural features of HS responsible for the binding of CyPB suggests the presence a specific 3O-sulfated N-unsubstituted glucosamine residue beside N-, 2O- and 6O-sulfates [210] (Fig. 3A). The presence of these interactions between HS and CyPB has been correlated to the cell type-specific expression of STs responsible for these modifications, including HS3STs [211], and showed a direct link between ST expression, HS sulfation pattern and CyPB-mediated cellular effects in T lymphocytes. As CyPB overexpression is currently associated with a poor prognostic and/or drug chemoresistance [207,208], targeting CyPB or blocking its interaction with HS could help to develop new anti-tumor treatments.

Important players of the immune response are the Natural Killer (NK) cells which recognize and destroy tumors in an antibody-independent manner. The regulation of NK cells is mediated by activating and inhibiting receptors on their cell surface. NK cell activating receptors include activating forms of Killer cell Ig-like Receptor (KIR) and Natural Cytotoxicity Receptors (NCR), NKp46, NKp44, and NKp30. Selective engagement of these receptors with cancer cell ligands can stimulate both cytotoxicity and cytokine production. NCR were reported to directly bind HS on cancer cells and to initiate tumor targeting by NK cells [212] (Fig. 3B). Each of these receptors recognized distinct HS structures with fine specificity. Both NKp30 and NKp46 bind to highly charged HS/Hep epitopes that are O-sulfated at C2 of IdoA and bear one to two sulfate groups on the glucosamine moiety [213]. A recent study revealed that the NK receptor KIR2DL4 directly interacted with HS/Hep and, importantly, that KIR2DL4 recognition of cell surface ligand(s) was regulated by HS3ST3B1 [214] (Fig. 3B). In agreement, HS3ST3B1 silencing by siRNA strongly reduced KIR2DL4 binding to pancreatic cancer cells PC3, supporting the contribution of 3O-sulfation in KIRDL4-HS interactions on the surface of NK cells. Altogether, these studies suggest that 3O-sulfation mediates direct interactions of NK cells and target cancer cells *via* their cognate receptors and regulates the trafficking of NCR and KIRDL4 to intracellular degradation or recycling pathways upon endocytosis of these receptors [214,215]. This opens up avenues towards treatments with Hep/HS analogs that would impact NK cell activity through binding to their cytotoxic cell surface receptors.

Additional studies reinforce the central role of HS3ST and 3O-sulfation in the control of NK cell recruitment and cytotoxicity. This process involves an alternative mechanism implicating Telomeric Repeat-Binding Factor 2 (TRF2), a subunit of the shelterin complex, which prevents inappropriate DNA damage response activation and protects telomere integrity (Fig. 3B). Biroccio *et al.* [216] showed that TRF2 binds an interstitial telomeric sequence (ITS) of *HS3ST4* gene and induces its expression, preventing the recruitment of NK cells. This group recently showed that TRF2 also triggered glypican-6 and versican expression and that induction of HSPG biosynthesis was associated with significant changes in the structure of the tumor microenvironment [217]. This results in the activation of Myeloid Derived Suppressor Cells (MDSCs) and acts as a general suppressor of the immune system by inhibiting NK and T cells. These results identify an immunosuppressive pathway for MDSCs, which links the TRF2 protein to glycocalyx reshuffling, suggesting novel strategies to prevent immune-surveillance escape and enhance the efficacy of cancer therapies.

There is increasing evidence that HS trigger immune activation through Toll-like receptor 4 (TLR4) and A Proliferation Inducing Ligand (APRIL) signaling during inflammation, and that this mechanism plays a part in the cancer process [179]. APRIL is a cytokine of the TNF family that acts through binding the cyclophilin ligand interactor (TACI) which is important in B lymphocytes biology and functions. It has been shown that APRIL is overexpressed in many cancer cells and tumors and promotes cell proliferation and tumor growth [218], which has been associated with a bad prognostic in several solid tumors [219]. APRIL specifically binds to HSPGs [220], a crucial event for APRIL-induced tumor growth [221]. A novel mechanism contributing to HS-APRIL collaboration in cancer was recently proposed [222]. In that model, HS released from breast cancer cells targeted TLR4 and RNA-activated protein kinase (PKR) to stimulate APRIL secretion by neutrophils, thus promoting cancer cell proliferation. Altogether, HS may exert a dual role (i) as a cell surface receptor or docking molecule for APRIL cytokine and (ii) as a ligand that induces APRIL secretion by neutrophils to induce cancer cells growth. This process would be favored by inflammation, which promotes neutrophil recruitment and induces HS fragment release in tumor microenvironment. No evidence of HS sulfation role in APRIL-HS interaction has been pointed out. However, as the HS sulfation pattern can directly influence HS functions, it could be possible to control HS fine structure and HS-cytokine interactions by modulating ST activities. Targeting sulfated HS (and indirectly cytokines which interact with these HS), could be a way to control cytotoxic immunity cells and to modulate their activation or inhibition in a

pathological context.

6. Concluding remarks and future prospects

We are entering an exciting period for tumor glycobiology, with bright future for the GAG field. Numerous significant studies underscore the crucial roles of HS and their biosynthetic enzymes as key regulators of tumor cell-ECM interactions governing cell signaling and tumor progression, and their significant prognostic value. For example, high expression of HS3ST3A is of poor prognosis in specific breast cancer subtypes [71].

In this review, we comprehensively discussed how cell-surface and matrix HSPGs modulate the activity of a myriad of soluble effectors including GFs, cytokines, chemokines and morphogens, either promoting or inhibiting their biological activity, and impacting progression of the malignancy. HSPGs act in cooperation with the receptors of these effectors, often by dimerization or oligomerization, in a way that is highly dependent on the cell and malignancy type and subtype [71,85]. HS, in the form of shed PGs or oligosaccharides, also act on plasma membrane receptors of companion cells present in the tumor microenvironment, such as stromal fibroblasts and immune cells [180]. These HS-mediated cell-cell communications have a great impact on the progress of cancer.

An unique feature of HS is their extraordinary pleiotropic actions. They act as general signal integrators to coordinate signaling and cell response, possibly *via* the formation of high HS oligomers that help to organize multimolecular complexes of proteins [11]. Finding out how HS sense and organize a variety of extracellular signals, to translate them into an appropriate intracellular signaling, is key to a better understanding of HS biology [163]. These issues cannot be fully examined in studies performed on the classically used cancer cell monolayer cultures. Implementation of novel approaches such as animal and patient-derived *in vitro* and *in vivo* models like organoids, which recapitulate key features of human tumors and specific characteristics of cancer subtypes, are valuable to gain a more global view of the unique pleiotropic and multi-directional mechanisms of action of HS. Such models will also be very helpful to test GAG-based drugs in preclinical tests and personalized medicine.

HS chains are not coded on a template like DNA or proteins, but result from a dynamic assembly line involving an array of biosynthesis and maturation enzymes, the expression of which is fine-tuned by different mechanisms to produce the desired binding motives for ligands. Epigenetic regulations appear most appropriate to integrate environmental factors and produce a fast response. Several studies, by us and others, show that HS3STs are particularly prone to epigenetic silencing, underscoring their capacity to sense and respond to the extracellular signals that they receive, and to act as key actors of the dynamic interplay between matrix, companion cells and cancer cells. What is the significance of these epigenetic regulations in the tumor evolution? Is the frequent hypermethylation of HS3ST2 a possible prognostic marker in cancer? Such questions need to be further explored.

The biological activity of HS chains primarily relies on their ability to bind a multitude of partners. AT-III, the prototypic ligand of 3O-sulfated HS and Hep, is quite peculiar. Its biological activity critically depends on binding to a Hep 3O-sulfated pentasaccharide with very high affinity, sufficient to induce a conformational change that activates AT-III. This discovery several decades ago, led to envisage a concept of one saccharide sequence binding to one protein ligand for achieving a given function [223]. Accumulating evidence now suggests that this may be correct only in few cases, notably involving 3O-sulfated HS motives. Indeed, information, in particular emerging from genetic studies in model organisms, supports the idea that specific 3O-sulfated HS motives establish selective and crucial interactions with several ligands including FGF, BMP, Notch, *etc*… [224]. Can we expect newly discovered 3O-sulfated ligands to show similar characteristics? Do they all mediate specific interactions with higher affinity than other modifications? These are intriguing questions.

Undoubtedly, technical issues have hampered faster progress in the investigation of the mysterious 3O-sulfation [105]. The small amounts of HS chains available from tissue sources make classical fractionation methods, like those that allowed the establishment of the structure of antithrombin-binding site in Hep, technically challenging. Most methods to characterize the 3O-sulfation modification are based on degradative techniques, which actually may have led to underestimating this modification [19]. The lack of defined standards is also a strong limitation for the precise estimation of 3O-sulfated oligosaccharides in cells and tissues, as well as for the evaluation of HS3ST activities. New analytical techniques, such as those based on tandem mass spectrometry-ion-mobility [225], two-dimensional liquid chromatography-mass spectrometry methods [226], as well as chemical and chemoenzymatic schemes to produce the much needed standards, should finally help to provide a more detailed view on the structures of 3O-sulfated binding sites [227,228], although this goal still requires much efforts.

Glycomic studies including glycoarrays dedicated to GAGs are expanded rapidly. Combined with other “omics” technologies, *i.e.* multiple unsupervised global transcriptome and proteome profiling approaches, they will have to be better harnessed to define the range of HS sequences, in particular 3O-sulfated, that function in conjunction with a particular signaling pathway. Advances in biochemical and global approaches should be synergistic to disclose the molecular mechanisms that governed 3O-sulfated epitope binding and function [229,230].

The 3O-sulfated HS field is expanding rapidly with puzzling findings that open exciting questions. Extracellular vesicles (EVs) and associated HSPGs have emerged as a central mechanism that coordinates the communication between stromal and cancer cells. Of note, EV uptake share pathways similar to the ones exploited by some viruses, in which 3O-sulfated epitopes are known to play an important role. The role of 3-sulfates in the HS pattern should thus be integrated with future studies on EV entry mechanisms [231]. In line with this, several studies recently reported various untypical locations of HS3STs and 3O-sulfated HS. An unexpected subcellular location of HS3ST2, in association with syndecan at the cell plasma membrane, was observed [73]. Interestingly 3O-sulfation and HS motives have been identified as critical for tau protein internalization [232]. By interacting with tau at the intracellular level, the 3O-sulfated HS produced by HS3ST2 may act as molecular chaperones allowing the abnormal phosphorylation of tau [233]. Hep and HS fragments, as well as shed syndecan, mediate the translocation of GFs, such as FGF and HGF to the nucleus [2], and mediate epigenetic regulation. All these various studies open up important area of investigations to answer questions such as: What is the role of HS3ST and 3O-sulfated HS in these unexpected locations? Might nuclear 3O-sulfated HS play a part in cancer? Linked to the previous issues, the concept of GAGosome has emerged, which suggests that HS biosynthesis enzymes function collectively within, probably dynamic, supra-molecular complexes. What is the role of the different HS3STs in the GAGosome?

The repertoire of ligands which bind 3O-sulfated HS is also steadily increasing as discussed in our review. It has to be underlined that their role should be carefully reexamined in view of improved methods, which may prove that 3O-sulfation has a more general contribution to various ligand binding sites, than currently considered. Linked to the discovery of these 3O-sulfated HS/Hep binding proteins, the variety of functions of 3O-sulfated HS is also increasing such as in immunity and in insulin regulation, amongst others. The implication of the modulation of the immune system is typically in the realm of the 3O-sulfated HS, and is one of the most exciting areas of current research related to the tumor biology.

As expected from such pleiotropic molecules, preclinical oncology studies revealed that HS-based therapeutics, including HS glycometics, can potentially affect all steps of cancer development and progression.

Their implication in modulating inflammation and immunity is also highly relevant to cancer therapeutics. The design of specific enzymes and effectors that can modulate HS sulfation patterns, in particular 3O-sulfation, is a most powerful and promising tool for the application of HS and their analogues as therapeutic agents. However, it is expected that the action of HS-based drugs would be context-dependent, depending on the type and even subtype of tumor [71]. As they are of good tolerability, they seem to be particularly appropriate to be included in conventional treatments to enhance their tumor efficacy [234]. A deeper comprehension of the beautiful world of HS, focusing on the 3O-sulfated epitopes, their incredibly pleiotropic effects, together with a better understanding of the regulation and function of the still intriguing HS3ST enzymes, should pave the way for an increasing further exploitation in cancer treatment and personalized medicine.

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