

Chapter 14

Involvement of Heparan Sulfate and Heparanase in Neural Development and Pathogenesis of Brain Tumors



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14.1 Malignant Brain Tumors

14.1.1 Incidence and Symptoms of Brain Tumors

Primary malignant central nervous system (CNS) tumors represent about 2% of all cancer types, although it accounts for high mortality rates [19, 102]. Brain malignancies are the leading cause of death from solid tumors in children and the third cause of death from cancer in adolescents and adults aged from 15 to 34 years [84]. Even though some tumors may be classified as benign, it can be deadly as a result of continuous growth and invasion into the confined space of the brain. Common symptoms and treatment options may vary depending on the tumor type, location, size and the age of the patient. Characteristic symptoms of brain tumors are headaches, vision problems, seizures, memory loss, and poor coordination. Meningiomas are the most common benign brain tumors, and gliomas that encompass the highly aggressive, grade IV GBM, are the most prevalent malignant brain tumors [19]. Primary brain tumors present a bimodal distribution, exhibiting a smaller peak in the pediatric population, at 5–9 years old, and a significantly higher number of affected individuals in the 60–69 age group [22]. The majority of the CNS tumors thus appear after 50 years of life, but also, they are the second most frequent cancer type in children between 5–15 years [22].

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14.1.2 Glioma and Glioblastoma

Malignant gliomas are the most common primary brain tumor. They often exhibit an infiltrative nature and resistance to radio/chemotherapy, as well as destruction of peri-tumor normal brain tissue. Grade IV glioma also called glioblastoma (GBM), is the most frequent and most malignant form of glioma, with only a 15-month median survival time for patients receiving standard therapy, i.e., maximal safe resection, followed by radiation and chemotherapy [172]. GBM is histologically characterized by diffuse infiltration, high cellular density, microvascular proliferation, and areas of necrosis surrounded by pseudopallasing cells. GBMs can be divided into primary and secondary GBM, where primary GBMs account for 90% and where the disease occurs without the existence of low-grade gliomas. Secondary GBMs, on the contrary, develop from lower grade glioma.

14.1.3 Genetic and Epigenetic Alterations in Glioblastoma

Development of GBM involves the accumulation of a large number of genetic and epigenetic alterations, such as point mutations, chromosome rearrangements, insertions, deletions, copy number alterations, and aberrations in DNA methylation as well as histone modifications. The loss of heterozygosity (LOH) in GBM is frequently found in chromosomes 1p, 10p, 10q, 13q, 19q, and 22q. LOH in 10q (47–70%), 10p (47%), *EGFR* amplification (36%), *p16^{INK4a}* (31%), *TP53* (28%), and *PTEN* (25%) are most frequently present in primary GBM. In secondary GBM, the most common alterations are LOH in 22q, *TP53* mutations (65%), and LOH in 19q (54%) and 10q (54–63%) [127]. Moreover, mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene have drawn attention as a novel paradigm in prognosis and is nowadays included in the diagnosis according to the WHO criteria [108]. This mutation was initially identified in integrated genomic sequencing, where it was found that recurrent mutations occur in the active site of *IDH1* in 12% of all GBM patients. *IDH1* mutations preferentially occur in younger patients, and in most patients with secondary GBM. Furthermore, patients with an *IDH1* mutation display a significantly favorable prognosis [130]. The cytosolic isocitrate dehydrogenase 1 protein encoded by *IDH1* is responsible for the reduction of NADPH, and the production of NADPH is essential for the regeneration of reduced glutathione, which eventually leads to resistance to apoptosis and protection against oxidative damages [95]. This may explain the elevated sensitivity to therapies in mutant *IDH1* cells.

Generally, GBM cells display global hypomethylation, but also regional hypermethylation at selected gene-associated CpG islands, that are un-methylated under normal conditions. Promoter hypermethylation frequently occurs in the *MGMT* (O⁶-methylguanine-DNA methyl-transferase) gene, *TIMP-3*, and *RB1* [127]. Histone modifications greatly influence transcription, and they are less stable than

DNA methylation and balanced by activities of histone-modifying enzymes, such as histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). Recurrent *H3F3A* mutations are prevalent in pediatric and young GBM patients [155], and different *H3F3A* mutations may suggest different cellular origins [173]. As an example of the relevance for histone modifications, the lysine-specific demethylase 1 (LSD1) was found to regulate the tumorigenicity of GBM by influencing the expression of Olig2, Sox2, and POU3F2 [91].

14.1.4 Aberrant Signaling Pathways in Glioblastoma

Studies of human GBM genomes and large-scale gene expression profiling have identified several signaling pathways commonly mutated in GBM. Primarily they are as follows: the PI3K-PTEN-AKT-mTOR pathway, the RAS/MAPK pathway, the TP53 pathway, and the RB pathway. These are key pathways controlling cell proliferation, survival, apoptosis, tumor angiogenesis, and metastasis.

The PI3K-PTEN-AKT and RAS/MAPK pathways are both induced by receptor tyrosine kinases (RTKs). Receptors of EGF and PDGF, two growth factors important in GBM tumorigenesis and CNS development, belong to RTKs and the amplification of the EGFR and PDGFR genes has been found in 13% and 45% of GBMs, respectively [1]. The activation of the RTK pathways subsequently initiates downstream effectors, such as PI3K and RAS. PTEN can antagonize the function of PI3K, and the mutation or deletion of PTEN has been reported in 38% of GBM [1]. Neurofibromin 1 (NF1) is a negative regulator of RAS [111], and NF1 mutations and deletions have been found in over 20% of GBM [1].

TP53 and RB are crucial regulators in cell cycle progression and tumor suppressor genes, frequently silenced in GBM. Normally, in the G₁ phase, pRB is inactivated by cyclin D/cyclin-dependent kinase 4/6 (CDK4/6)-induced phosphorylation, which leads to a release of E2F and the subsequent entry into the S phase. p16^{INK4a} is an inhibitor of cyclin D/CDK4/6, and p14^{INK4a} neutralizes MDM2, an E3 ubiquitin ligase negatively regulating TP53. They are both encoded by *CDKN2A* and are frequently homozygously deleted in GBM (2008). TP53 can bind to the promoters of over 1000 downstream genes, including p21, which subsequently blocks the cell cycle and initiates programmed cell death.

14.1.5 Molecular Classification of Glioblastoma

Glioblastoma is characterized by an extreme inter- and intra-tumoral heterogeneity [25]. The Cancer Genome Atlas research network (TCGA) has provided molecular classification of adult GBMs based on their gene expression and mutational profiles. Hence, defined molecular GBM subgroups have been identified [181] [189]. The Proneural (PN) subtype is associated with PDGFRA, IDH1, and TP53 mutations.

High expression of PDGFRA, NKX2-2, and OLIG2, i.e., genes associated with oligodendrocyte development is a typical feature of PN GBM. A subset of younger GBM patients with PN tumors exhibits global hypermethylation (termed glioma-cytosine-phosphate-guanine-CpG island methylator phenotype, G-CIMP) [16] and have better prognosis than G-CIMP negative tumors. However, comprehensive DNA methylation profiling in a large cohort of glioma patients shows that the relation of G-CIMP and IDH1 status is not always correlated [16].

The Classical subtype (CL) is characterized by amplification of EGFR homozygous, deletion of the Ink4a/ARF locus and lack of TP53 mutations. In addition, neural stem and progenitor cell markers were highly expressed in CL GBM. As mentioned above, loss of MGMT and incapacity of this enzyme to repair the mismatch increases the sensitivity to TMZ. MGMT-methylated tumors of the CL subgroup respond better to temozolomide as compared with non-MGMT-methylated CL GBMs [124].

The mesenchymal (MES) subtype is characterized by hemizygous NF1 deletion and low levels of NF1 mRNA. Mesenchymal markers (CHI3L1/YKL40, MET) and astrocytic markers (CD44, MERTK) are high in the MES subtype. Several markers of the MES subtype are shared with the EMT process [203]. There is a high degree of necrosis and inflammation in MES GBMs and an expression signature of wound healing, and NF- κ B target genes [181].

14.1.6 Medulloblastoma (MB)

Medulloblastoma is the most common form of malignant embryonal pediatric brain tumor. It is believed to arise from granule neuron progenitors (GNPs) or other undifferentiated stem or progenitor cells in the cerebellum, in or near the brain stem [55, 199]. MB have traditionally been divided by histopathology; classical MB (> 70% of cases) characterized by dense small round cells with large nuclei and small cytoplasm. The desmoplastic/nodular MB (D/N) (~20% of cases) were named due to the high connective tissue content in the internodular regions. The anaplastic/large cell MB (LC/A) (~5% of cases) with a poorer prognosis has been defined by round cells with prominent nucleoli, numerous mitotic figures and apoptotic bodies [38, 72]. MB with extensive nodularity (MBEN) shows a better prognosis [138].

14.1.7 Molecular Subtypes of Medulloblastoma

Studies over the last ten years have revealed, distinct molecular MB subtypes that are related to clinical outcome. This has, at least to some extent, enabled patient-specific treatment options, sparing children with the most benign variants from extensive treatment, thus reducing some treatment-related side effects. MB has been recently further categorized into subtypes of the already existing subgroups that

reveal intertumoral heterogeneity, by combining expression and methylation data analysis of patient samples [23]. This allows further categorization of MB tumors and provides prognostic information and risk stratification. Very recent single-cell transcriptomic studies demonstrate different cellular origins for the different molecular subtypes, mirroring the different cell populations in the developing cerebellum [182].

The WNT subgroup has the most favorable prognosis among all the subgroups and represents 10% of all MBs [188]. Approximately all the WNT MBs are identified by activation of the WNT signaling pathway, usually caused by activating mutations in the beta-catenin (CTNNB1) gene resulting in stabilization of the protein [40]. WNT MBs have been further classified into WNT α and β [23].

The SHH subgroup has an intermediate prognosis and SHH MBs represent 30% of all MBs; with 5-year survival ranging between 60–80% [166]. The most frequent alterations in SHH MBs appear in the SHH pathway components, mainly patched1 (PTCH1), suppressor of fused (SUFU) but also focal amplifications of MYCN and GLI2 [188]. Also, mutations of TP53 are found in childhood SHH MBs and are in more than half of these cases associated with germline TP53 mutations from the Li-Fraumeni syndrome and have poor outcome. Additionally, SHH MB has recently been further classified into 4 different subgroups. The SHH α subtype is defined by TP53 mutations, MYCN and GLI2 amplification associated with a very poor prognosis in children, while SHH β MBs are relatively metastatic, thus resulting in poor outcomes in infants. The SHH γ subtype is characterized by better outcomes in infants without signature mutations in comparison to SHH δ subtype, which frequently contains TERT promoter mutations and defines mostly adult patients [23].

The Group 3 subtype of MB is the most aggressive and invasive of the four subgroups and represent approximately 25% of all MBs [166]. Group 3 tumors are often located in the fourth ventricle near the brainstem, but as they show a very invasive phenotype, almost 50% of Group 3 patients display metastasis at diagnosis [143]. There is amplification of MYC in approximately 20% of cases in this subgroup. Notch and transforming growth factor beta (TGF- β) signaling pathways have also been found altered in Group 3 MBs [89, 125]. Recent integrative analysis suggests 3 subtypes in Group 3 MB: Group 3 α tumors which exhibit metastasis at diagnosis; Group 3 β have a high frequency of GFII activation and orthodenticle homeobox 2 (OTX2) amplification and Group 3 γ , often exhibiting MYC amplification with an invasive phenotype at diagnosis [23].

The Group 4 subtype has an intermediate prognosis and comprises 35% of all MBs [166]. They are often located into the fourth ventricle near the brainstem and are commonly metastatic at diagnosis. Frequent changes in Group 4 MBs include inactivating mutations in the histone lysine demethylase gene KDM6A, gene duplication of synuclein-alpha interacting protein (SNCAIP) as well as MYCN and CDK6 gene amplification [116]. Group 4 tumors can be further subdivided into 3 subtypes: Group 4 α usually has focal CDK6 amplification, chromosome 7q gain, 8p loss and MYCN amplification. Despite the fact that Group 4 γ exhibits similar mutation profile, it does not have MYCN gene amplification. Finally, Group 4 β is augmented in SNCAIP and PRDM6 overexpression [23].

14.2 Cancer Stem Cells

14.2.1 *The Concept of Cancer Stem Cells*

The regulation of stem cell number in tissues needs to be highly controlled since mutations affecting stem cells may result in uncontrolled proliferation, and ultimately the development of cancer. The concept of cancer stem cells (CSCs) describes the stem cell-like cell of origin, which is believed to initiate tumor formation (reviewed in [28]). At the same time, the term cancer stem cell is also used for those rare self-sustaining cells in a tumor that have properties such as the specific ability to resist irradiation and chemotherapies, not shared by the bulk of tumor cells [109]. This preserves dormant cancer stem cells, that can seed a new tumor and are believed to be responsible for relapse after therapy. Cancer stem cells thus possess a unique capacity for growth and progression and are probably responsible for relapse. The cancer stem cell hypothesis predicts that solid tumors have a hierarchical organization, where CSCs drive tumor maintenance and recurrence. Tumor expansion would thus be the result of unlimited ability for self-renewal by CSCs that are more resistant to chemotherapy and irradiation, than the majority of tumor cells. Therefore, unless the CSCs are targeted, cancer treatment will not be successful. Multiple studies have described this concept for malignant brain tumors, both those affecting the adult population [164] and pediatric brain tumors [68]. Over the last decade, attempts have been made to define characteristics and markers of brain tumor stem cells (reviewed in [99]) but the hypothesis has been challenged by the concept of intrinsic plasticity driving tumor potential in a non-hierarchical manner [34].

14.2.2 *Models of Cancer Stem Cells from Brain Tumors*

Building on the view that CSCs constitute only a marginal part of the total tumor, studies of whole tumors are not the best model of the CSC niche, and therefore a reason to culture CSCs separately. Furthermore, if the hierarchical structure of CSCs is correct, new CSC clones with different genetic alterations may emerge over time due to selection and genomic instability, giving rise to tumor heterogeneity. This underscores the need for large numbers of cell lines for each tumor type. Finding new drugs and drug combinations that target the CSCs remains an unmet medical need. Development of drugs for this purpose has been hampered by the lack of valid cell models [101] and cancer drug screens have relied on serum-cultured cell lines. GBM cells can be expanded using neural stem cell culture conditions [139], and we have established a panel of clinically annotated and experimentally validated cancer stem cell lines from GBM [195]. This resource, termed the Human Glioma Cell Cultures (HGCC), is a collaborative effort to provide newly established and well-characterized cell lines derived from GBM patient tumor tissue. The

HGCC cell lines which have been established and cultured under stem cell conditions are available as an open resource (<http://www.hgcc.se>) along with accompanying data for research and drug discovery.

Serum-free culture conditions are presumed to preserve characteristics of the original tumor, but it has proven more challenging to propagate medulloblastoma than GBM, using neural stem cell culture conditions. This is illustrated by the observation that classical medulloblastoma cell lines, established in the 1980s are still the most prevalent cell culture methods for this disease (reviewed in [80]). The difficulty in establishing patient-derived medulloblastoma cell lines also skews *in vitro* studies because the WNT and Group 4 subtypes, while comprising half of the cases, are very scarcely represented when it comes to cell lines. A recent serum-free protocol that employs high-adherence plastic for monolayer culture, rather than sphere formation, showed improvement in success rate [152]. Alternatively, unmanipulated, human medulloblastoma cells can be propagated as xenografts, retaining stem cell-like properties [32].

14.3 Heparan Sulfate and Heparanase in Neural Development

14.3.1 *Heparan Sulfate and Heparanase in Development*

Heparan sulfate proteoglycans (HSPGs) are composed of a core protein onto which highly charged sulfated saccharide chains are attached. They interact with a large number of physiologically important molecules. The major enzyme that degrades HS is heparanase (HPSE), an important regulator of ECM remodeling that has been shown to promote the growth and invasion of several cancer types. The crucial role of heparan sulfate (HS) in mouse development has been demonstrated by a number of mutational studies on HS biosynthesis and modification enzymes. The deletion or deficiency in enzymes required for biosynthesis initiation and elongation leads to almost a complete lack of HS that causes severe phenotypes. GlcAT-1 knockout mice showed embryonic lethality before the 8-cell stage because of failed cytokinesis [82]. Mice deficient in EXT1 failed to gastrulate and generated smaller embryos due to defects in forming organized mesoderm and extra-embryonic tissues [106]. Mice with the complete depletion of EXT2 exhibited phenotypes similar to EXT1-deficient mice. Although, the heterozygotes had a normal life span and were fertile, they displayed multiple abnormalities in cartilage differentiation [171] and failed to respond properly to FGF signaling [159]. NDST enzymes define the basic sulfation state of HS chains and NDST1 deficiency resulted in neonatal lethality due to a condition resembling respiratory distress syndrome [146]. Disruption of C5 epimerase led to perinatal lethality, with renal agenesis, lung defects, and skeletal malformations [104]. Depletion of uronyl 2-O-sulfotransferase/glucosaminyl 6-O-sulfotransferase-1/glucosaminyl 3-O-sulfotransferase-1 led to perinatal,

embryonic, and partial lethality, respectively [20, 62, 162], while depletion of XylT2, NDST2, SULF1, and SULF2 in mice only caused mild phenotypes [4, 29, 45, 96]. However, SULF1/SULF2 double mutant mice exhibited delays in myogenic differentiation and regeneration after skeletal muscle injury [97], and NDST1/NDST2 double-knockout embryos died as early as E3.5 [71].

The importance of HSPGs in neural development is well established, for example, in axon guidance. Mice with the conditional knockout of EXT1 in nestin-positive cells showed severe guidance errors in major commissural tracts [77]. Complete loss of HS2ST or HS6ST1 led to axonal navigation errors in retinal ganglion cells [140] and severe corpus callosum phenotypes via the alteration of ERK signaling [27]. In addition, HS has also been shown as a requirement for neural progenitor cell proliferation via modulating cell signaling. For instance, the loss of HS2ST resulted in a significant proliferation reduction in cerebral cortical precursors [113]. Ablation of perlecan in the developing mouse brain led to decreased delayed cell cycle progression in neural progenitors due to altered SHH and FGF2 signaling [56]. Furthermore, syndecan-1 knockdown reduced neural proliferation via modulating response to WNT ligands [190].

Heparanase (HPSE) is the predominant degradation enzyme for HSPGs. It is an endo- β -glucuronidase that cleaves the β -1,4-glycosidic bond between D-glucuronate and D-glucosamine, liberating fragments between 4 to 7 kDa [185]. The active form of HPSE is secreted, and it acts on the cell surface and ECM, releasing HS-binding molecules and disassembling the ECM in association with cell migration and tissue remodeling. There are also reports suggesting the nuclear translocation of HPSE during cell differentiation [88] and in tumor cells [123]. Under normal conditions, HPSE is expressed in platelets, mast cells, placental trophoblasts, keratinocytes, and leukocytes. In pathological conditions, such as inflammation, atherosclerosis, and cancer a marked elevation of HPSE expression is frequently observed [183, 184] (Vlodavsky et al., Gaskin et al., Ilan et al., Chaps. 1, 7 and 9 in this volume).

Mouse strains overexpressing HPSE or that are devoid of HPSE have been generated. Somewhat surprisingly, neither of these mouse strains exhibits severe phenotypes; they are fertile and have a normal life span, without prominent functional or pathological alterations. HPSE knockout mice exhibited an accumulation of long HS chains and showed a marked elevation in matrix metalloproteinase (MMP) [201]. HPSE-overexpressing mice had a profound decrease in HS chain length and exhibited a reduction in food consumption and an accelerated hair growth rate. Also, they showed increased levels of urinary proteins, enhanced neovascularization, and disruption in epithelial basement membranes [202]. There is very little information about HPSE in brain development, but the levels have been reported to be highest during early postnatal development, especially in the neocortex [121]. The same authors found that there is differential expression between different regions of the brain, and in the neocortex, the amount of enzymatically active HPSE decreases sharply after the first two weeks after birth.

14.3.2 Heparan Sulfate-Dependent Signaling in the Neural Stem Cell Niche

The neurogenic and tumorigenic niches are similar and we have reported that the composition of the extracellular matrix (ECM) of the former undergoes developmental changes [13]. Heparan sulfate proteoglycans (HSPGs), main components of the niche modulate the activities of other factors, e.g. growth factors (reviewed in [197]). A vital role for HS biosynthesis in neural stem cell differentiation has been reported [46]. One crucial role of HS is to function as a co-receptor for growth factors on the cell surface. The mechanism of HS-dependent signaling was first found and has since been extensively studied, with regard to FGF2. HS chains increase the binding affinity of FGF to its receptor [142, 200] and play an essential role in ligand-receptor binding kinetics [47]. A similar signaling model was later described in other pathways, including BMP, WNT, SHH, PDGF, and VEGF signaling [2, 42, 54, 114, 144, 150, 177].

Besides regulating cell signaling, HSPGs have multiple functions in cell physiology. They transport chemokines across cells and present them on the cell surface. Serving as a component of the ECM, HS chains facilitate cell-ECM interaction and cell adhesion via cooperation with integrins and adhesion receptors. As receptors for proteases and their inhibitors, HS chains regulate their activity and spatial distribution. Altogether, HSPGs have the potential to manipulate major processes in the body and therefore have important implications in normal stem cell differentiation, development, and pathological conditions. HPSE, by virtue of cleaving HS, can modulate these signaling cascades, for example, HPSE is necessary to sustain FGF-2 signaling in epithelial-mesenchymal transition of proximal tubular epithelial cells to form myofibroblasts [112]. Moreover, FGF2-signaling in melanoma cells is modified by HPSE [145].

14.3.3 Heparan Sulfate and Heparanase in Stem Cell In Vitro Differentiation

Although the crucial role of HSPGs and their modification enzymes in embryonic development has been demonstrated in a series of mouse models, the severe phenotypes of the animals prevent further study on their functions in mouse nervous system development (see above). Instead, using ES cell differentiation *in vitro*, the function of HSPGs in stem cell commitment and differentiation can readily be evaluated. Moreover, in normal ES cell differentiation, the regulations of N-, 3-O-, and 6-O-sulfation have been observed [86]. ES cells exhibit a low level of N-sulfation and increased expression of NDST4, HS3STs, and HS6STs during differentiation to NSPCs [120]. During differentiation from neuroepithelial precursors to neurons, the cells distinctly changed their 6-O-sulfation pattern and HS chain length [18]. These discoveries suggested a role of sulfated HS in stem cell differentiation. When ES

cells are differentiated using a monolayer differentiation protocol [30] they first go through an expansion of NSPCs, followed by differentiation of NSPCs into mature neural lineages, i.e., neurons and glia. In our own studies, we observed that during the expansion phase, HPSE mRNA increases dramatically, followed by a gradual decrease during final differentiation. The latter coincides with a rise in the amount of HS during a phase when NSPCs are maturing into neurons and glia. Thus, the expression of HSPE is reduced, while the quantity of HS increases during neural differentiation [196].

To understand the role of HS and HPSE in neural differentiation, ES cells with deletions or overexpression of biosynthetic enzymes and modifying genes have been used. The complete knockout of EXT1 causes absence of HS chain synthesis, which has severe consequences for neural differentiation. EXT1-knockout ES cells had phenotypically normal colonies and a high expression of pluripotent markers, but depletion of EXT1 led to a differentiation arrest when subjected to monolayer differentiation [86, 92]. Although EXT1-knockout ES cells could form embryonic bodies, they could not generate terminally differentiated cells [70]. When directing these ES cells to neural differentiation, the addition of soluble heparin could partially rescue differentiation to mature neurons [86]. In another ES cell line, using the knockdown of EXT1 with short hairpin RNA, soluble GAGs were capable of inducing neural differentiation via influencing various RTK pathways [137]. NDST1/NDST2 double-knockout ES cells were completely devoid of N-sulfation but retained a very low level of 6-O-sulfation [71]. Similar to EXT1-knockout ES cells, NDST1/NDST2 double-knockout ES cells maintained a normal phenotype and pluripotency in a culture. However, they generally failed to differentiate upon embryoid body formation [98]. Angiogenic sprouting could occur in NDST1 / NDST2-deficient embryoid bodies, but the adhesion of pericytes to nascent sprouts was reduced, owing to the dysregulation of transforming growth factor beta and PDGFB signaling [100].

When using stepwise protocols, by allowing the ES cells to first differentiate to multipotent progenitors, surprisingly, the NDST1/NDST2 ES cells were able to give rise to osteoblasts, albeit with lower efficacy than wild-type ES cells, but no adipocytes were generated [46]. Under conditions inducing neural differentiation, these ES cells appeared to be blocked at a primitive ectoderm-like state, expressing the early ectodermal marker FGF5 without proceeding to neural progenitors. However, the differentiation to neural precursors could be restored by a combination of heparin and FGF2 or FGF4, but this only succeeded in a very narrow concentration range [46].

Studies of ES cells overexpressing HPSE have shown that they possess a faster proliferation potential, and they also formed larger teratomas *in vivo*, than their wild type counterparts. This faster growth rate was kept during differentiation, as monitored by the monolayer protocol for neural induction, and they also show enhanced activation of ERK and AKT pathways [196]. Interestingly, neural progenitors overexpressing HPSE differentiated to a larger extent into oligodendrocytes, than wild type ES cells that hardly generated oligodendrocytes at all, and this increase was at the expense of neurons that were reduced, while the proportion of astrocytes did not

change [196]. This shows that alterations in HS levels and composition can change how the stem cells use various signaling pathways and consequently alter their differentiation potential.

14.4 Heparan Sulfate and Heparanase in Cancer Stem Cells

14.4.1 *HS, HPSE and Cancer Stem Cells*

Cancer stem cells recapitulate many characteristics of normal stem cells. Early studies showed that several stem cell differentiation programs depend critically on an adequately modified HS, for example in myoblast differentiation [142] and hematopoietic stem cells [60]. As already mentioned, loss of function studies for HS biosynthetic genes have shown their critical role in vertebrate development since HS2ST or NDST-1 knockout mice die in the neonatal period [20, 146]. As described above, intriguingly, HPSE knockout mice do not display any major phenotypic disturbance, and there are no reports of affected stem cell pools when HPSE gene is lacking, although their mammary glands displayed a more abundant branching compared with glands from wild type mice [201]. In addition, *in vivo* neovascularization in a matrigel plug was pronounced in HPSE knockout mice, and *ex vivo* sprouting assays revealed an increased sprouting [201]. It has also been reported that the function and activation of macrophages are hampered in HPSE null mice [61] as they express lower levels of cytokines and exhibit reduced mobility. If, and how, any of the above alterations relate to effects on stem cell pools, or their progeny, remains to be investigated.

Several studies have shown that properly sulfated HS is required for ES cells to switch from self-renewal to initiation of differentiation of specific cell lineages [92], for instance, to capillary structures [83], or neural progenitors [86]. During ES cell differentiation, HS of the more differentiated progeny become more complex and increasingly sulfated [137]. Stem cell differentiation thus relies on correctly sulfated proteoglycans, and cancer stem cells, in contrast, would carry HS with a lower degree of sulfation, which endows them with survival advantages. This is corroborated by the higher expression of HPSE in cancer stem cells, e.g., in breast cancer [76], and glioblastoma [93]. Another example of how deregulated proteoglycans influence cancer stem cells is that serglycin, normally found in the secretory granule of hematopoietic cells, when overexpressed, serves as a marker of poor prognostic in lung cancer. Here, serglycin was reported to enhance stemness properties by induction of NANOG expression in NSCLC [59].

14.4.2 HPSE in GBM Stem Cells

Using patient-derived glioblastoma stem cell cultures [195], we have shown that HPSE is highly expressed, compared to normal brain, and that both the latent 65-kDa and the enzymatically active 50-kDa forms can be detected [93]. That HPSE produced by GBM stem-like cells was functional could be determined by reduced cell numbers upon either shRNA downregulation of HPSE, or by treatment with the HPSE inhibitor PG545. In an attempt to determine if HPSE expression could be associated with specific features of GBM, we found that Mesenchymal GBM cells express the highest levels of HPSE when compared to primarily the Classical subtype [93]. This was confirmed in tumor tissue when we analyzed the TCGA dataset, where expression of HPSE was highest in the Mesenchymal subtype and, therefore, it seems plausible that HPSE expression reflects GBM heterogeneity. Heparanase-overexpressing glioma cells were also more resistant to stress and chemotherapy [161], a well-described feature of cancer stem cells.

14.5 Heparan Sulfate and Other Proteoglycans in Brain Tumors

14.5.1 ECM Remodeling as Part of the Brain Tumor-Supporting Microenvironment

Less attention has been paid to the brain tumor ECM compartment, than to the cancer cells and non-tumor cells of the tumor microenvironment. The ECM of the normal brain is distinct from other organs and consequently, the brain tumor matrix is different from that of other solid tumors. Any tumor stroma outside the brain is usually rich in fibrillar collagens, while in the CNS, glucosaminoglycans, proteoglycans and glycoproteins are predominant constituents. ECM molecules are highly functional entities in almost every aspect of brain tumor biology, in addition to their anchoring and organizing functions. Taking up between 10 and 20% of the volume of the brain [15], the ECM molecules thus not only provide structural support but are also part of signaling systems that can be co-opted by the brain tumor to enhance cancer cell proliferation, invasion, vascularization, immune infiltration, etc. There are many ways by which proteoglycans can support malignancy of the brain tumor microenvironment and thereby contribute to the failure of clinical trials. For example, HPSE is increased in glioblastoma stem cells [93], and abnormal receptor tyrosine kinase activity is a common denominator of GBM [181]. Since extracellular availability of growth factors is orchestrated by e.g. HSPGs, excess HS degradation by HPSE is a way by which brain tumors could modify the microenvironment to drive oncogenic signaling.

14.5.2 Characteristics of the Extracellular Matrix in the Brain

The adult brain ECM can be described in three compartments: that of the neural interstitial matrix, i.e., (i) ECM molecules in the parenchyma, (ii) the basement membrane ECM, and (iii) the perineuronal nets. The ECM of the brain parenchyma consists mainly of networks of hyaluronan and proteoglycans, which are produced intracellularly and then secreted into the extracellular space [15] where it surrounds cells and attaches to the cell membrane [11]. Other components are glycoproteins such as tenascins and to a smaller extent, collagens, laminin, and fibronectin. The basement membrane surrounds the pial surface and forms a barrier between the vasculature and the parenchyma. It mainly contains collagen IV, laminins, fibronectin, dystroglycan, and heparan sulfate proteoglycans, e.g., in the form of perlecan [79]. Finally, the perineuronal nets are mesh-like structures of proteoglycans, tenascin R, and link proteins around neuronal cell bodies [94]. The role of perineuronal nets is to stabilize synapses and therefore, they are important in regulating CNS plasticity [187].

For normal development to proceed, and to prevent aberrant remodeling in the adult brain, ECM components are strictly regulated during neurogenesis, differentiation, neural migration and axonal outgrowth [9]. ECM molecules that are abundant during embryogenesis and early postnatal development regain expression levels in glioma, for example, tenascin-C [52, 53, 69]. It is well established that ECM molecules contribute to the extrinsic regulation of the local microenvironment of neural stem cell niches in the brain [43]. This regulation occurs at several levels including adhesion to other cells of the niche. Mechanical properties of the ECM results in different matrix stiffness which influences stem cell fate, and stem cell-ECM interactions mediate different signaling events.

14.5.3 Analyzing Proteoglycans in Brain Tumors

An early study showed that high-grade glioma cells in culture, to a larger extent than normal cells, produce HS and release GAG chains into the cell culture medium [169]. The same authors demonstrated this as a diffuse and intense staining of HS which was localized to the surface of the cell, in contrast to normal cells or low-grade astrocytoma that displayed punctate HS staining. Bertolotto et al. [14] investigated surgical specimens of human glioma and normal brain, and found very high glucosaminoglycan levels, particularly heparan sulfate and dermatan sulfate in GBM, compared to normal brain.

A multidimensional mapping of specific proteoglycans of brain tumors remains to be presented. A GBM cohort has been analyzed (The Cancer Genome Atlas, TCGA) for RNA expression of proteoglycan core proteins, biosynthetic and modifying enzymes [186]. The authors found several of these genes to be differently

expressed, both when comparing tumors to non-neoplastic tissue controls, and also between GBM subtypes.

Recent approaches for analyzing proteoglycans in brain tumors include mass spectrometry and Raman microspectroscopy. The latter was used in a recent study [90] and presents identification of proteoglycans based on their vibrational signatures. For this proof of principle paper, a medulloblastoma specimen was investigated and proteoglycans were found to be deregulated. Liquid chromatography-mass spectrometry analysis was employed in a study by Tran et al. [178] to profile HS disaccharide content and structure across patient-derived sphere cultures of GBM cells. The authors found significant heterogeneity in the HS content and structure between patients, and suggested that the intertumoral differences in proteoglycan expression could be analyzed to determine which tumors would more likely respond to HSPG modification.

14.5.4 Examining the Cancer Genome Atlas for Proteoglycans with Deregulated Expression in Glioblastoma Patients

Overall, many genes that had previously been reported to promote cancer progression, such as those involved in metastasis, were among the most highly regulated genes revealed upon examination of the TCGA cohort [186]. Both membrane-bound and secreted proteoglycans are, in general, more highly expressed in GBM than normal brain tissue, which could suggest proteoglycans and their synthesizing and degradation enzymes as new cancer biomarkers for GBM. CSPG4, also denoted NG2, was first identified as a marker of oligodendrocyte precursor cells [122] and its overexpression has been detected in glioma [160]. Furthermore, oligodendrocyte precursors have been identified as one type of glioma-initiating cells [107], and due to its overexpression in a vast majority of GBM cases, NG2 may have prognostic value [175]. Protein tyrosine phosphatase receptor β/ζ (PTPRZ1) is also highly expressed in the TCGA cohort. It has been associated with glioma formation and recently, a small molecule inhibitor of PTPRZ1 was found to inhibit glioma formation in vivo [50]. A very high expression of CD44 was also noted [186], which is in line with CD44 being reported as overexpressed in glioma, especially in the mesenchymal subtype [134] and commonly used to enrich for cancer stem cells [5].

Out of the modular proteoglycans, versican showed the highest expression in GBM [186], and other studies confirm high levels in mouse and human glioma of this secreted proteoglycan [75]. Another study showed that antibodies to versican could reverse the migration-promoting effect of TGF- β 2 on glioma [7]. Furthermore, versican was found, among other ECM genes, to be part of a signature for invasiveness of low-grade astrocytoma [151].

Among HSPG core proteins that have been reported to be altered in glioma, are glypican-1 [174] and syndecan-1, the latter shown to be upregulated via NF κ B activation [191]. Syndecans 2, 3 and 4 are ubiquitously expressed in normal brain and

glioma [191], whereas syndecan-1 is not detected in the normal brain. In a study of over 100 glioma samples, high syndecan-1 expression correlated to shorter survival, and grade IV patients had the highest expression [198]. Several small leucine-rich proteoglycans are highly expressed in GBM, among them fibromodulin. Fibromodulin was identified in a screen of epigenetically regulated genes in GBM and found to be an essential regulator of glioma cells [115].

14.5.5 Heparan Sulfate and Chondroitin Sulfate Biosynthetic Enzymes in Glioblastoma

During HS biosynthesis, the nascent HS chain is modified by several enzymes, in the order as follows: *N*-deacetylase/*N*-sulfotransferase, C₅-epimerase, 2-*O*-sulfotransferase, 6-*O*-sulfotransferase, and 3-*O*-sulfotransferase. The NDSTs define the design of sulfation patterns, which in turn dictate the affinity for different ligands [31]. For the enzymes that synthesize HS and CS, there is a wide variation in mRNA expression in GBM. Five of the CS biosynthetic enzymes are upregulated, and the other four downregulated [186]. No comprehensive public data set link specific CS biosynthesis genes to brain tumor development or progression, but the role for CSPG in glioma has been studied [163]. Silver et al. report that intense staining of CSPG was seen around non-invasive gliomas, similar to that described for brain injury, where CSPG has repulsive actions [153]. Furthermore, there was very little glycosylated CSPG in xenografts of diffusively infiltrative glioblastoma [163]. This may seem somewhat in disagreement to some reports of upregulated core proteins in brain tumors, but when the level of glycosylation, rather than the expression of core proteins, was analyzed the former seems to be the determining factor as to whether CSPG promotes or inhibits invasion. In another study, “underglycosylated” brevican was associated with late stages of glioma progression, such as invasion, whereas it did not affect glioma stem cells [37]. To date, there is not enough data to conclude precisely how CS biosynthetic genes contribute to malignant brain tumors.

Understanding if the biosynthetic mechanism of HS is altered in glioblastoma is critical for determining the roles for HSPG in brain tumors. Therefore, it is interesting to note that according to TCGA data, all four NDST genes were downregulated in GBM, thereby suggesting less elaborate sulfation of HSPG in GBM [186]. A vast majority of the HS biosynthetic genes are down-regulated in GBM (12 out of 15 genes in TCGA). HS3ST3a1 was among the three upregulated genes and is highly expressed in glioma. HS3ST3a1 was the predominant sulfotransferase in glioma cells, which is not the case in normal human astrocytes [174]. TCGA revealed low expression of all 6O-sulfotransferases, and higher expression of two out of four 3O-sulfotransferases, which could indicate that low 6O-sulfation is a feature of GBM. This is supported by RT-PCR on a cohort of glioma patients of different

grades, where both grade III and grade IV gliomas had lower expression of HS6ST1 and HS6ST2 than non-tumor tissue from the same patient [180].

14.5.6 Heparan Sulfate Modifying Enzymes in Glioblastoma

Once the HS chain has been completed it can be further edited by sulfotransferases, SULF1 and SULF2, both of which are up-regulated in GBM [186]. SULF2 removes 6-O-sulfate moieties and thereby activates several signaling pathways. As mentioned, a common denominator of glioma is the abnormal tyrosine kinase activation, and because HS-GAGs have a negative charge, they can bind many growth factors and thus play a key role in RTK activation. A typical example hereof is PDGFRA, which is often amplified in GBM, and PDGF ligands are frequently expressed at high levels in this tumor. PDGF is considered a driver gene in glioma and has been shown to cause glioma in mice (reviewed in [158]). Phillips et al. [135] described SULF2 overexpression in human GBM and cell lines derived from GBM patients, and showed that knockdown of SULF2 led to smaller tumors in mice. This corresponded to HSPG-dependent signaling by PDGFRA, presumably through increasing the availability of growth factors in the tumor microenvironment. The effect was most notable in the proneural subclass of GBM, which is primarily driven by PDGF signaling, but not in classical GBM where perturbed EGFR signaling is a key feature. Furthermore, the importance of SULF2 in glioma is supported by the gene being identified through insertional mutagenesis in retrovirus-driven PDGF-induced mouse glioma [85].

14.5.7 Heparanase in Glioma and Medulloblastoma

HPSE, as described, is the main HS degrading enzyme, which releases HS-bound bioactive molecules and thus primes the tumor microenvironment to support cancer spread. HPSE is implicated in metastasis and invasion of many types of cancers [183] and has also been reported to be overexpressed in GBM [74, 93] and medulloblastoma [165, 168]. Fig. 14.1 summarizes the current knowledge about HPSE in brain tumors, and its complex route of activation. First, the pre-pro-HPSE is directed to the ER lumen via its signal peptide. The 65 kDa, latent form of HPSE is then transferred to the Golgi apparatus, and then into secretory vesicles that bud off from the Golgi. When HPSE reaches the outside of the cell, it interacts with HSPGs and quickly taken up again as a complex with HSPG into early and late endosomes. Next, the endosomes fuse with lysosomes and HPSE is activated, upon cleavage by Cathepsin L, to the 50 kDa enzymatically active form. The lysosomal HPSE can take different routes, either secreted to perform extracellular functions, but also translocate to the nucleus (Ilan et al., Chap. 9 in this volume). Our own recent finding shows that HPSE can stimulate signaling pathways by interacting with CD24 [10].

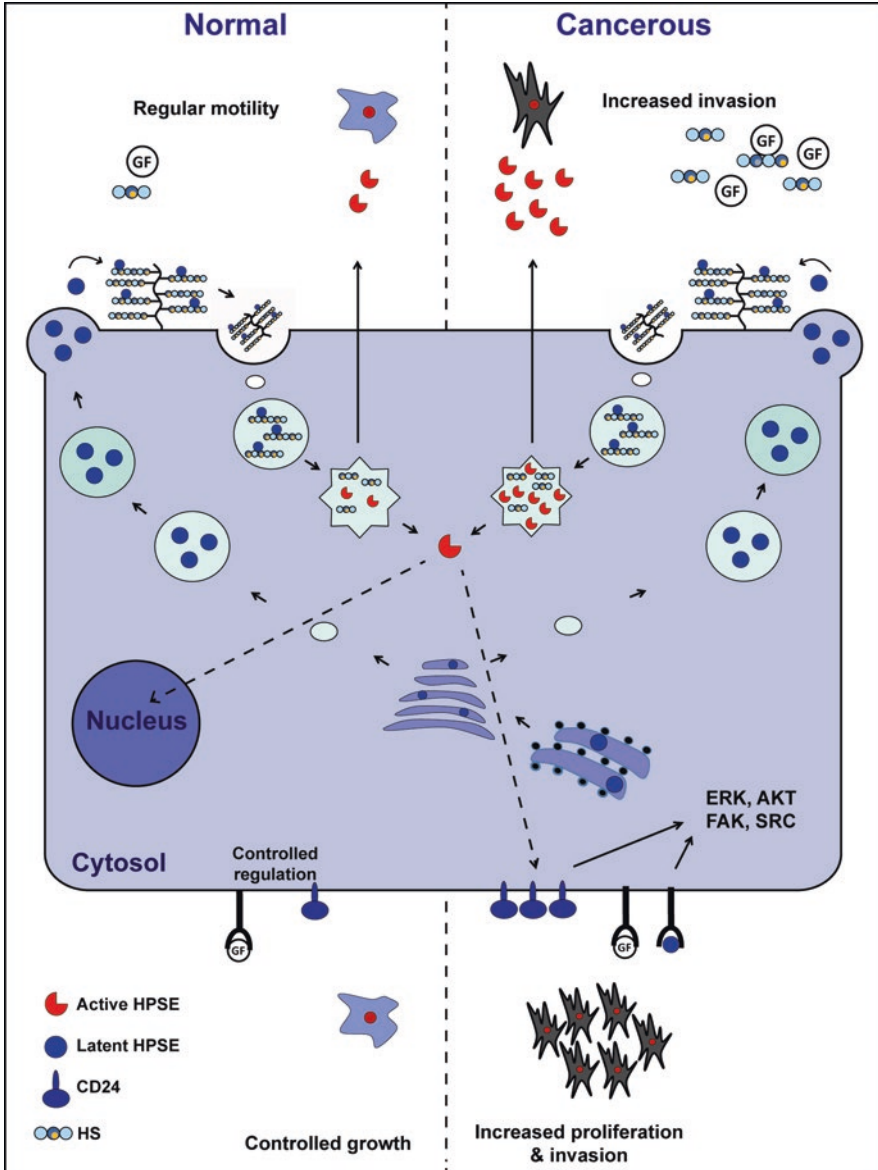


Fig. 14.1 HPSE in normal and cancerous tissues, e.g. brain tumors, and the complex process of HPSE activation. Upon synthesis, the pre-pro-HPSE is first directed to the ER lumen via its signal peptide. The 65 kDa latent form of HPSE is then transferred to the Golgi apparatus, and then into secretory vesicles (light blue) that bud off from the Golgi. When HPSE reaches the outside of the cell, it interacts with HSPGs and rapidly taken back again into early and late endosomes as a complex with HSPG. Next, the endosomes fuse with lysosomes (star-shaped vesicle) where HPSE is activated, upon cleavage by Cathepsin L, to the 50 kDa enzymatically active form. The lysosomal HPSE can take different routes, either secreted to perform extracellular functions and/or also translocate to the nucleus. Our recent finding shows that HPSE can also stimulate signaling pathways by interacting with CD24

Hong et al. described increased levels of HPSE mRNA in glioma, compared to normal brain, but could find no correlation to the WHO malignancy grade, when comparing oligodendroglioma, anaplastic astrocytoma and GBM [74]. The expression data were confirmed by western blotting and immunostaining, and by transplanting human GBM-derived spheres to immune-deficient mice. In contrast, in another report, HPSE was not detected in human GBM, and following injection of U87 GBM cells into the brain of immunologically compromised mice, HPSE expression in these cells disappeared [179]. The histological staining of glioma patient tissue was performed with two different HPSE antibodies for these two studies, something that might explain the contradictory data. Using the U87 cell line, other investigators showed that modest over-expression of HPSE in U87 cells enhanced tumor size after xenografting, but not the level of HPSE expression [204]. Possible explanations for the above discrepancies could be the use of different cell lines, i.e., patient-derived cells cultured using serum-free stem cell conditions [74] versus U87 [179], which is a classical serum-cultured glioblastoma cell line. Another study revealed that when HPSE was overexpressed in U251 GBM cells, it led to increased invasion, colony formation, and AKT phosphorylation [73].

We found strong overexpression of HPSE in glioma patients, using a cohort of 182 glioma patients with different WHO-grade tumors, and report low-grade gliomas to be less intensely stained by anti-HPSE antibodies than high-grade gliomas in the neuropil [93]. Also, we reported that down-regulation of HPSE reduced GBM proliferation *in vitro*, while the addition of HPSE enhanced cell growth, and activated ERK and AKT signaling [93]. Based on our data obtained using HPSE transgenic or knockout mice, we reported that the HPSE host brain level affects tumor size. We suggest that surface-associated or secreted HPSE promotes the invasive properties of high-grade gliomas and, consequently, enhance tumor progression by HPSE residing in the microenvironment. Also, in pediatric brain tumors, we detected high HPSE levels compared to non-tumor brain, and when treating pediatric brain tumor cells (medulloblastoma and other embryonal tumors) with HPSE their growth was stimulated [168], as was the case with glioblastoma.

Furthermore, we found for both glioma and medulloblastoma that the latent 65 kDa form of HPSE which requires intracellular processing to become active, rapidly activates the ERK and AKT signaling pathways, before we could detect any enzymatically active HPSE [93, 168]. Therefore, the mechanisms for HPSE action in brain tumors could be both enzymatic and non-enzymatic. To study the underlying mechanisms of HPSE in brain tumors, we used inducible U87 glioblastoma cells for overexpression of HPSE. Differential expression analysis identified CD24, a mucin-like cell adhesion protein, as upregulated by both active and enzymatically inactive HPSE [10]. Patients who express high HPSE and CD24 had a shorter survival time than those who had high HPSE and low CD24 levels. When overexpressing CD24, it stimulated glioma aggressiveness *in vitro*, and tumor growth as xenotransplants, which could be blocked by anti-CD24 or anti-HPSE antibodies. Furthermore, antibodies to L1CAM, one of the CD24 ligands, also attenuated the

tumors in vivo [10]. Our results thus describe a new HPSE-CD24-L1CAM axis at work in glioma tumorigenesis.

When we used an inhibitor of HPSE, PG545, it efficiently killed pediatric brain tumor cells, but not normal human astrocytes, suggesting specificity to cancer cells that express high levels of HPSE [168]. The compound PG545 also inhibited tumor cell invasion in vitro and very potently reduced flank tumors in mice. Taken together, findings from several laboratories, including our own, indicate that HPSE in malignant brain tumors affects both the tumor cells themselves and their microenvironment. Thus, HPSE plays a substantial role in the progression of brain tumors and may represent a therapeutic target.

14.6 Heparanase Inhibition as a Novel Brain Tumor Therapeutics?

14.6.1 *Rationale for Heparanase Inhibition*

Based on a vast literature, it is clear that HPSE contributes to tumor progression, which has led to an interest in targeting the enzyme for therapeutic purposes. Mostly, HS mimetics have been suggested as potential inhibitors of the enzyme, although many different aspects have to be considered. HS mimetics vary in size and kinetics and have different efficiencies against HPSE [64]. As HPSE is typically not highly expressed in normal tissue, side effects to inhibition should be manageable.

14.6.2 *Low Molecular-Weight Heparin*

Heparin or heparin-derivatives such as low molecular-weight heparin (LMWH) have been suggested for cancer treatment, but its contribution to survival improvement is not clear. [8, 48]. Enoxaparin decreased the growth of non-small cell lung cancer [3] and, similar to tinzaparin and dalteparin, has been shown to reduce FGF-induced mitogenesis through ERK kinase inhibition in endothelial cells [167]. Tinzaparin and unfractionated heparin (UFH) decreased metastases in colon adenocarcinoma and melanoma cell lines [170] and inhibited endothelial tube formation, VEGF expression, and angiogenesis [117, 118]. Most of the antitumor effects of heparin-like derivatives are the result of sequestering and blocking growth- and angiogenic- promoting factors [48, 110]. There are clinical studies where LMWH has been given to GBM patients, but without significant prolonged survival, although a trend was noted (reviewed in [154]). In the Eastern Cooperative Oncology Group clinical trial, dalteparin was tested for potential overall survival benefits, in combination with radiation therapy, but this study was closed early, due to the introduction

of temozolamide [148]. Another randomized placebo-controlled trial included newly diagnosed WHO grade 3 or 4 glioma patients, who were given long term dalteparin, but this trial was also closed early, partly due to bleeding [132]. In a third study, GBM patients were treated with enoxaparin using 1 and 2-year overall survival as main endpoints, and progression-free survival as an additional endpoint. Here, there was a significant benefit for the 13 patients on LWMH compared to the control 17 patients at one year, but not at two years follow-up [207].

14.6.3 PI-88 (*Mupafostat*)

PI-88 (*Mupafostat*) is a mix of highly sulfonated mannan oligosaccharides [44]. PI-88 exerts its anti-angiogenic and anti-metastatic properties by inhibiting HPSE and blocking interactions of FGF-1/2 and VEGF with their receptors [129]. In preclinical studies, PI-88 reduced the invasion and metastasis of rat adenocarcinoma cells [129] and decreased leukemic cell burden in mouse models [81]. It also inhibited late-stage tumor growth and early progenitor lesions in a pancreatic mouse model, and that was linked to a decrease in cell proliferation, angiogenesis, and increased tumor apoptosis [87]. PI-88 is the most well-studied heparan sulfate mimetic in clinical trials to date, through several phase I and II trials for patients with hepatocellular carcinoma (HCC) [12, 26, 103]. A phase III trial as adjuvant therapy was initiated for patients with HCC, but this trial was lately canceled upon interim analysis [157] (Chhabra and Ferro, Chap. 19 in this volume). No clinical trials for PI-88 have, to date, been registered in clinicaltrials.gov for brain tumors.

14.6.4 SST0001 (*Roneparstat*)

SST0001 (*Roneparstat*) is a modified glycol-split heparin, which is fully N-acetylated and hence exert little or no anticoagulant activity [147]. SST0001 inhibits HPSE enzymatic activity and displays a decreased capacity to release ECM-bound FGF-2 in comparison to unmodified heparin. In multiple myeloma cells, SST0001 inhibited HPSE and expression of HGF, VEGF, and MMP-9, resulting in decreased angiogenesis. It also inhibited HPSE-mediated degradation of syndecan-1, which enhances myeloma cell proliferation [147]. SST0001 (=Roneparstat) reduced the growth of disseminated myeloma tumors in vivo when combined with conventional chemotherapy [141]. SST0001 has been examined in clinical trial for multiple myeloma and is documented to be safe at a dose of 200 mg/day [51] (Noseda and Barbieri, Giannini et al., Cassinelli et al., Purushothaman and Sanderson, Chaps. 12, 15, 21 and 23 in this volume). No trials on brain tumor patients have been initiated.

14.6.5 M402 (*Necuparanib*)

M402 (necuparanib) is an N-sulfated glycol-split (GS) modified heparin. It has the advantageous properties of a heparan sulfate-like molecule but was specially engineered to considerably decrease anticoagulant activity. Hence, it has been used alone and/or combined with standard chemotherapy, and it showed substantial anti-metastatic activity in preclinical models [205]. A clinical phase I/II intervention trial was started for M402, combined with nab-paclitaxel and gemcitabine for the treatment of metastatic pancreatic cancer but terminated due to insufficient efficacy. It has not been tested against brain tumors.

14.6.6 PG545 (*Pixatimod*)

PG545 (Pixatimod) is a synthetic, single molecular entity fully-sulfated tetrasaccharide [36]. In comparison to many other HS-mimetics, its structure allows enhanced pharmacokinetic properties and decreased anticoagulant properties [35]. PG545 is a highly effective inhibitor of HPSE compared to the other HS mimetics used to date [64]. In pancreatic cancer cell lines, it inhibited Wnt/b-catenin signaling and reduced the proliferation of tumor cells by the proangiogenic growth factors VEGF, FGF-1, and FGF-2 [36]. PG545 has been studied in multiple preclinical models in various tumor subtypes, exhibiting potent antitumor, anti-metastatic, and anti-angiogenic effects [36, 63, 128, 193]. Importantly, PG545 is the only HS mimetic investigated so far that has an immune-stimulatory effect and exerts its major anti-lymphoma effects through activation of the immune system via natural killer (NK) cells [17] (Bendersky, Yang and Brennan, Chap. 18 in this volume). PG545 was tested in a Phase 1a study to establish the maximum tolerated dose for patients with advanced solid tumors, and although it gave some adverse effects, such as fever and elevated triglycerides, PG545 is considered to have a safety and pharmacokinetic profile that merits further development [65] (Hammond and Dredge, Chap. 22 in this volume). We have found a very good inhibitory effect of PG545 both *in vitro* and *in vivo* (mouse) in GBM [93] and pediatric brain tumors [168], but due to lack of ability to cross the BBB, PG545 in its present form would be difficult to develop for brain tumors.

14.6.7 Small Molecule Approaches to HPSE Inhibition

Low molecular-weight inhibitors against HPSE are still lacking, but with the 3D structure of HPSE being resolved [194] (Wu and Davies, Chap. 5 in this volume), hopefully, future efforts of designing new inhibitors as therapeutic agents will be more successful (Giannini et al., Chap. 23 in this volume). Another approach could be metallo-shielding of HS by polynuclear platinum complexes that are positively charged, as these would mask the ECM-resident HS from HPSE action [133].

14.7 Challenges to Brain Tumor Treatment

14.7.1 *Invasiveness*

CNS tumors are different from other malignancies due to their location, and they rarely metastasize outside of the brain, even though they rapidly invade the surrounding brain parenchyma. An invasive and aggressive growth pattern is a feature of malignant brain tumors, but their invasiveness is different from other malignant solid tumors that commonly extravasate into the blood and lymphatic vessels. In the brain, motile glioma cells can take several routes, along blood vessels, following white matter tracts, or, diffusively in the brain parenchyma [57]. A recent study of human GBM using radiology, suggests that white matter tracts are the preferred direction for human GBM invasion, possibly due to their anatomical features [41]. Invasive tumor cells cannot be removed at initial surgery, and therefore contributes to the fatal outcome by seeding new tumors.

14.7.2 *Heterogeneity*

Brain tumors exhibit extensive inter- and intra-tumoral heterogeneity [49, 78, 116] which arises from expansion of clones carrying different mutations [37]. Intra-tumoral heterogeneity leads to selective pressure, either by clonal evolution or by chemo- and/or radiotherapy. Hence, the resistant clones remaining after therapy will be the ones forming the recurrence, thus creating a secondary tumor [126], which is further diversified by treatment-induced mutations. Indeed, GBMs constantly evolve so that within one GBM tumor several subtypes can co-exist and individual cells within the same patient exhibit a spectrum of expression profiles, which leads to selection of tumor subclones [131]. This extreme heterogeneity is challenging for GBM drug discovery because a candidate drug response may vary widely between cell lines from different GBM patients, and even in clones from the same patient due to plasticity [156].

14.7.3 *The Blood-Brain Barrier*

One of the most challenging aspects for brain tumor treatment is the blood-brain barrier (BBB) which limits the entry of therapeutic molecules into the brain [39]. It consists of tight junctions that seal off the brain endothelial cells in order to protect the brain from the crossing of unwanted endogenous and exogenous particles. The complex vasculature of the BBB, compared with blood vessels in the rest of the body, serves as a major obstacle to successful therapeutic agent delivery to the

brain [39, 119]. Though advanced stage brain tumor may compromise the BBB to a certain degree, it is still not possible for most drugs to penetrate [136]. Attempts are progressing to be able to successfully target brain tumors, and invent novel CNS delivery systems for future clinical application, such as pulsed ultrasound [21]. As described earlier in this chapter, our efforts to deliver PG545 to block HPSE action in orthotopic brain tumors yielded no detectable drug in the brain tissue, despite the advanced stage glioma [168].

14.7.4 Drug Penetration in Brain Tumor Tissue

Chemotherapeutic agents against malignant brain tumors have been disappointingly inefficient, partially due to their unsuccessful accumulation across the tumor mass [58]. This is not only due to the inability to cross the BBB. Another challenge is the existence of a blood-tumor barrier (BTB) [24]. The BTB is considered a barrier because it is composed of small microvessel populations that block the access of large-drug molecules into the tumor [58] and is different from the BBB. BTB microvessels have high expression of drug efflux transporters [6, 176] and ABC transporters [105] compared to normal brain. Furthermore, GBM blood vessels have several features that compromise their functionality, including a high degree of microvascular proliferation and thus, even if a drug is delivered to the brain tumor, distribution across the tissue is hampered by the highly abnormal GBM vasculature [33].

14.8 Summarizing the Role of Heparanase for Brain Tumor Hallmarks

Systematic description of the so-called hallmarks of cancer provides a conceptual overview of principle ways for cancer cells to overcome the protective functions of the host organism [67]. This includes the specific capacities of the tumor microenvironment, as outlined by Hanahan and Coussens [66]. In this chapter, we have reviewed how HSPG and HPSE can contribute to the malignant brain tumor phenotype. Below, and in Fig. 14.2, we summarize the specific role of HPSE for some brain tumor hallmarks.

14.8.1 Promoting Proliferation

Several studies across many cancer types have shown that an increase in HPSE correlates to an increase in cell number, but the mechanisms behind augmented amounts of cells could be either increased cell proliferation, decreased cell death, or

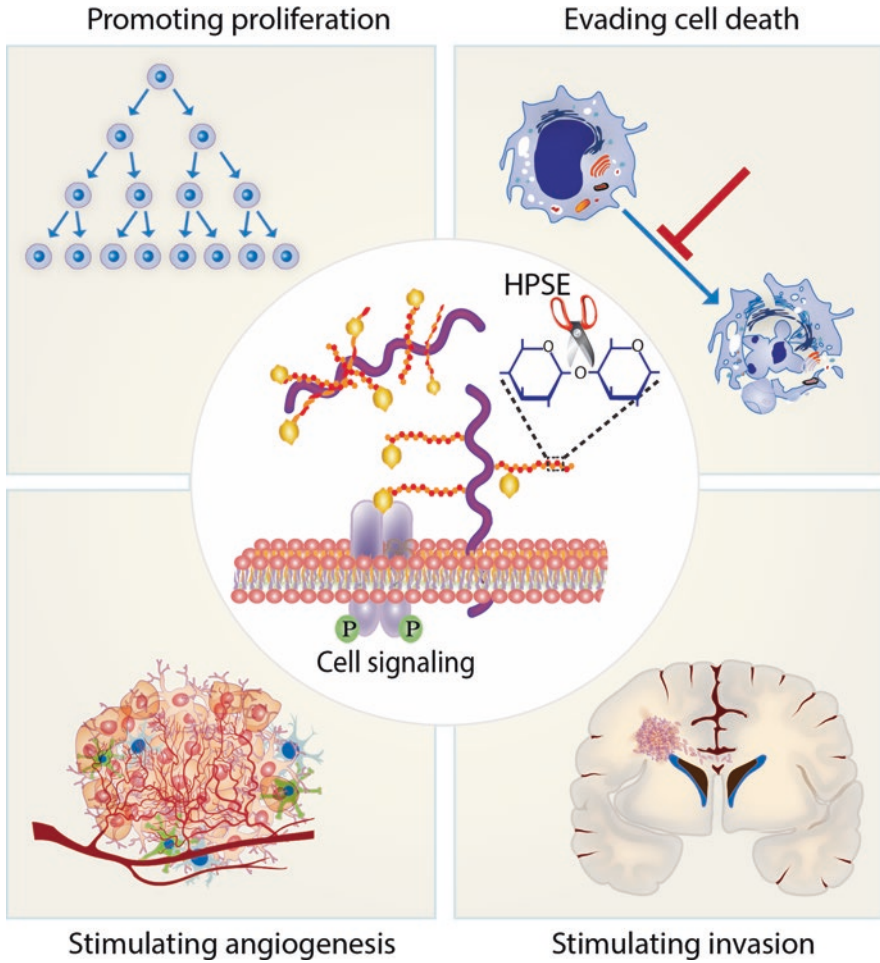


Fig. 14.2 Role of HPSE for brain tumor hallmarks

Promoting proliferation: In many cancer types, including brain tumors, overexpression of HPSE correlates with increased cell number, and this could be due to augmented cell proliferation, a decrease in cell death, or combination of both. Ki67 staining and BrdU incorporation have shown that proliferation is among the effects by HPSE on brain tumor cells.

Evading cell death: The finding that HPSE inhibition induced massive expression of cleaved caspase 3 in flank tumors of medulloblastoma indicates that HPSE is involved in suppressing apoptosis.

Stimulating angiogenesis: HPSE has been shown to stimulate brain tumor angiogenesis. One way to exert this function may be through the release of angiogenic factors, such as VEGF that are bound to HSPG in the ECM.

Stimulating invasion: Several studies show that HPSE increases brain tumor cell motility as revealed by scratch assays, chemotaxis assays, and invasion assays through Matrigel, or collagen gels. These studies also revealed activation of signaling pathways that are commonly associated with cell migration and invasion

a combination of both. In their study, Hong et al. show that overexpression of HPSE in U251 cells leads to an increase in cell growth compared to control cells, as measured by the MTT assay [73]. On the other hand, Zetser et al. reported a decrease in proliferation, as measured by BrdU incorporation in U87 cells overexpressing HPSE, compared to the parental cell line [204]. In GL261 mouse glioma, addition of recombinant HPSE or conditioned medium from HPSE-expressing cells increased the cell number and activated ERK and AKT pathways, and inhibition of HPSE by shRNA or HPSE inhibitor PG545 reduced cell numbers both in GL261 cells and patient-derived GBM cells [93]. The same results were obtained for medulloblastoma and another embryonal tumor [168]. Blocking HPSE was also found to reduce pediatric brain tumor cell proliferation in vivo [168] and HPSE overexpressing GBM cells had higher numbers of Ki67-positive cells than non-HPSE expressing tumor cells [74].

14.8.2 Evading Cell Death

Cancer cells have developed several mechanisms to overcome cell death, such as avoiding apoptosis or the ability to adapt to hypoxia. That HPSE is involved in suppressing apoptosis was suggested by the finding that HPSE inhibition induced massive expression of cleaved caspase 3 in xenografts of medulloblastoma [168]. Albeit a different brain tumor, pituitary tumor cell culture viability was decreased when HPSE was inhibited [149] further underscoring the role of HPSE in tumor cell survival. In order to withstand stress, caused for example by cytotoxic agents such as chemotherapy, cancer cells may also induce autophagy, an evolutionary conserved and important homeostatic cellular recycling mechanism [192]. HPSE can enhance the stress-resistance of GBM cells by increased autophagy in HPSE-overexpressing cells [161].

14.8.3 Stimulating Angiogenesis

Several studies show that HPSE stimulates brain tumor angiogenesis. For example, a GLI splice variant, TGLI1, has been shown to support glioma primarily through neovascularization, and that this effect is mediated by HPSE and VEGF-A [206]. In orthotopic glioma, tumor vascularization, as measured by CD31 staining in the peritumoral area, was enhanced in HPSE-Tg mouse brain compared to HPSE-KO brain [93] and in xenografts of medulloblastoma HPSE inhibition greatly reduced CD31 staining [168].

14.8.4 *Stimulating Migration and Invasion*

Most of the studies addressing HPSE in glioma report an increased migration and invasion as a consequence of overexpressed HPSE. In U87 cells, overexpression of HPSE leads to faster migration that covered the empty area in a scratch assay and to a more pronounced invasion through Matrigel [204]. Likewise, an increase in the number of U251 cells towards a chemotaxis gradient and increased invasion was reported for U251 cells overexpressing HPSE [73]. Similarly, a massive reduction of invasion in collagen gels, and attenuated migration in scratch assays were noted with pediatric brain tumor cells in response to HPSE inhibition [168].

14.8.5 *Concluding Remark*

In conclusion, our review of the current literature suggests that an improved understanding of the biology HSPG biosynthesis and degradation, particularly the involvement of HPSE, should have implications on designing therapeutic approaches towards treating GBM and medulloblastoma. Up to date, few drug targets have been directed towards ECM molecules in the brain tumor microenvironment, and therefore, validating the efficacy of inhibiting HS turnover as a potential therapy to brain tumors is highly warranted.

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