HEPARAN SULFATE PROTEOGLYCANS AND THEIR ENDOGENOUS MODIFYING ENZYMES: CANCER PLAYERS, BIOMARKERS AND THERAPEUTIC TARGETS

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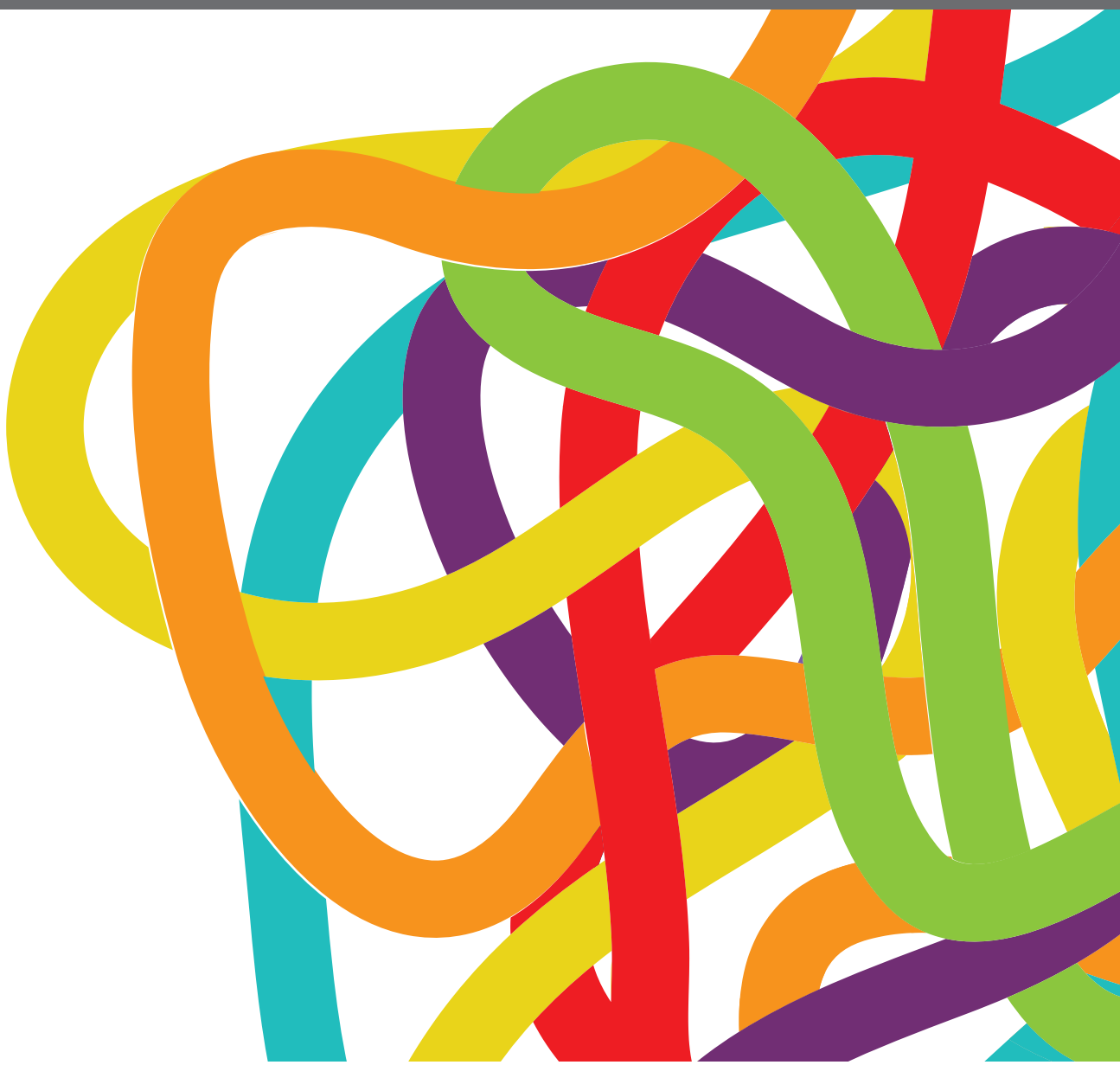

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HEPARAN SULFATE PROTEOGLYCANS AND THEIR ENDOGENOUS MODIFYING ENZYMES: CANCER PLAYERS, BIOMARKERS AND THERAPEUTIC TARGETS

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Editorial: Heparan Sulfate Proteoglycans and Their Endogenous Modifying Enzymes: Cancer Players, Biomarkers and Therapeutic Targets

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Editorial on the Research Topic

Heparan Sulfate Proteoglycans and Their Endogenous Modifying Enzymes: Cancer Players, Biomarkers and Therapeutic Targets

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Heparan sulfate proteoglycans (HSPGs) are glycoproteins ubiquitously expressed at the cell surface and in the extracellular matrix (ECM). The coordinated action of several heparan sulfate (HS) biosynthetic (e.g., sulfo-transferases) and modifying enzymes (e.g., heparanase, sulfatases) provides these molecules with a marked structural diversity and a peculiar ability to interact with a plethora of biomolecules through HS chains. Dysregulation of HSPGs has been associated with tumor pathogenesis and progression. Moreover, altered expression or deregulated function of HSPG biosynthetic/modifying enzymes has been implicated in key processes including proliferation, angiogenesis, metastasis, and drug resistance. Exploitation of the broad potential of HSPGs and related enzymes as biomarkers and therapeutic targets requires in-depth understanding of the context-dependent and, in some cases, contradictory roles of these molecules in tumors and their microenvironment. This aspect is highlighted by authors in the present Research Topic where mechanistic insights into the multifunctional roles of HSPGs and related enzymes in cancer and immune regulation are provided with a focus on cell signaling, structural issues, and therapeutic implications.

An overview of alterations that characterize the progressive destruction of the normal ECM leading to establishment of a cancer-permissive microenvironment, is provided by Elgundi et al.. Underlying the role of HSPGs in the various stages of the metastatic process, they focus on perlecan, a basal membrane HSPG that may exhibit opposing functions related to either the cellular context or its modular structure (Elgundi et al.).

Special attention has been dedicated to glypicans (GPCs), a subgroup of cell membrane HSPGs predominantly expressed during embryonic development in a strictly regulated way, while being undetectable in most adult tissues. GPCs regulate relevant morphogenic signaling pathways, including those involving Wnts, Hhs, BMPs, and FGFs. Seminal studies in hepatocellular carcinoma (HCC) provided the rationale endorsing the development of GPC3-targeted immunotherapy. GPC3, expressed in over 80% of HCCs, emerged as an actionable therapeutic target as well as useful prognostic biomarker. The review of Kolluri and Ho addresses the role of glypican in regulating HCC cell signaling. GPC3 forms a complex with both Wnt and the Frizzled receptor, activating canonical Wnt/β-catenin signaling. Moreover, GPC3 rescues circulating Wnt providing ligand storage at the cell surface. The activity of the 6-O-sulfatase Sulf-2, overexpressed

in 60% of HCC, contributes to the release of HS-stored Wnts further promoting Wnt signaling activation. Furthermore, in cooperation with the transcription co-activator Yap, also overactivated in HCC, GPC3 may contribute to the development of liver malignancy by modulating the Hippo pathway (Kolluri and Ho).

Immunotherapeutic strategies developed to target GPC3 include peptide vaccines, immunotoxins, monoclonal and bispecific antibodies, cytotoxic T lymphocytes (CTLs), and engineered T cell therapies. Codrituzumab (GC33), an anti-GPC3 recombinant humanized mAb and ERY974, an anti-GPC3/CD3 bi-specific T cell-redirecting antibody, are currently under clinical evaluation in HCC patients. Shimizu et al., report results from early clinical trials conducted by their team with HLA-restricted GPC3 peptides indicating that these vaccines can improve prognosis without eliciting non-specific autoimmune responses in most HCC patients. Encouraging results with GPC3 peptide vaccine were observed in patients with HCC and in GPC3-positive advanced ovarian clear cell carcinoma in Phase II studies (Shimizu et al.).

GPC3 is highly expressed in a variety of pediatric solid embryonal cancers including hepatoblastomas, Wilms and rhabdoid tumors, germ cell tumor subtypes and a minority of rhabdomyosarcomas. Ortiz et al., highlight that, although clinical trials demonstrated the safety and potential benefit of GPC3-targeting strategies in adult patients, evaluation of these immunotherapies in pediatric patients may be more challenging considering the distinct physiological pattern of GPC3 expression in infants in liver and kidney. T cells genetically engineered with a GPC3-CAR (GAP T cells) and a GPC3 peptide vaccine are currently under clinical investigation in pediatric patients with GPC3-positive solid tumors. Next generation GPC3-targeting approaches such as TCR-engineered T cell therapy are also under development offering potential therapeutic options for these patients (Ortiz et al.).

In pancreatic cancer, GPC1 expression significantly correlates with pathologic grade and clinical stage, and is closely associated with poor prognosis. Wang et al., address mechanisms altering GPC1 expression in cancer including DNA hypomethylation, microRNA expression and KRAS mutation, and examine the role of glypican in mediating key cellular signaling in tumorigenesis and angiogenesis. The authors also review studies investigating the potential of circulating GPC1 as a cancer biomarker and discuss possible reasons that may account for the contradictory results reported previously (Wang et al.).

Sulfation degree and pattern heavily impact the HS interactive abilities and functions. Denys and Allain summarize the emerging evidence for a role of the 3-O-sulfotransferases (HS3STs) in cancer. By catalyzing glucosaminyl-3-O sulfation, they produce rare HS modifications which affect the selective binding of several ligands. Altered expression of HS3STs has been associated with tumor-promoting or tumor-repressing effects depending on cellular and environmental context, substrate specificity and subcellular distribution of the enzymes, availability of acceptors or compensatory expression of HS3ST isoenzymes. Epigenetic repression of the HS3ST2, HS3ST1, HS3ST3A1 family members was found to be associated with the progression of several malignancies. In contrast, overexpression of HS3ST3B promoted epithelial-to-mesenchymal transition and angiogenesis in pancreatic and lung cancer, likely by favoring ligand binding to cell surface HS and activation of NRP1, VEGF, and TGF-β signaling. The design of specific HS3ST inhibitors is expected to provide insights into the role of the enzymes in cancer and the opportunity of modulating HS 3-O-sulfation to improve therapies (Denys and Allain).

A challenging goal in this field is to determine how the HS glycoside sequence and sulfation pattern drive ligand binding specificity. Brunetti et al., address this issue using the tetrabranched peptide NT4 which selectively binds HS on the tumor surface. The authors found a correlation between NT4 cell binding and basal expression of Sulf-1 and−2, suggesting that peptide binding was affected by HS 6-O-sulfation. Moreover, investigation of structural determinants of HS binding sites suggested multivalent binding of NT4 to densely sulfated clusters and a higher affinity for GPC3 and GPC4 among HSPGs. In addition to be useful probes for structural studies, as cancer selective HS-targeted agents, NT4, and possibly newly designed peptides, exhibit a theranostic potential since they can be conjugated with various functional units for drug delivery or tracer transport for tumor imaging (Brunetti et al.).

Among HS modifying enzymes, heparanase, the only HS specific mammalian endo-β-D-glucuronidase, was the first to be investigated as a cancer drug target. Heparanase plays a well-recognized role in inflammation, tumorigenesis, cancer progression, and drug resistance. Coombe and Gandhi revisit the milestones of the heparanase discovery, re-examine its role as a cancer-associated and metastasis promoting enzyme, and discuss its multiple non-enzymatic activities in light of structural data. Early observation of the potent heparanase inhibitory activity of heparin paved the way for the screening of heparin/HS mimetics as heparanase inhibitors. Based on promising preclinical data, some of them are currently under clinical investigation although none has been approved yet. The authors comment on the difficulty of interpreting data with HS mimetics due to their pleiotropic effects including immunomodulation. The multifunctional activity of heparanase, its subcellular and extracellular localization and internalization mechanisms, as well as its contribution to physiological processes, are additional aspects that need to be clarified to fully understand the potential of heparanase, a valid but challenging target, according to the authors. They also point out the potential influence that the closely related heparanase 2 and the T5 heparanase splice variant, both lacking catalytic activity, may exert on *in vivo* efficacy of anti-heparanase drugs (Coombe and Gandhi).

An emerging area of clinical interest is the heparanase contribution to immune regulation. The production of heparanase by tumor and/or stromal cells (e.g., leukocytes) can result in mutual influence on gene expression and phenotypic behavior. The relative contribution of the enzyme from different cellular sources and the underlying molecular mechanisms are just beginning to be elucidated. Mayfosh et al., provide an overview of the current knowledge of heparanase expression and functions in leukocytes highlighting its two-sided role. Novel

leukocyte-based anticancer therapies e.g., CAR-T cell therapy, dendritic cell vaccines and viral-therapeutic delivery exploiting heparanase are under development. The emerging picture is that the choice of the appropriate therapies inhibiting pro-tumorigenic or promoting anti-tumorigenic effects of heparanase will depend on a better understanding of the particular cancer setting. For instance, heparanase inhibitors may have more chance of being effective for malignancies in which leukocyte-derived heparanase promotes tumor progression such as colorectal and pancreatic carcinoma (Mayfosh et al.).

By applying CRISP-Cas9 technology and lentiviral cell infection to stably knock down or overexpress heparanase in colorectal cancer models, Liu et al., demonstrate that the endoglycosidase promoted tumor growth and liver metastatic dissemination. Transcriptome analysis confirmed the link between heparanase and genes/pathways involved in ECM remodeling. Among these, the metalloproteinase MMP1 was shown to be positively regulated by heparanase via p38 MAPK signaling (Liu et al.).

By using a mouse model of metabolic syndrome/diabetes and concurrent pancreatic ductal adenocarcinoma (PDAC), Goldberg et al., reveal a new mechanism underpinning the preferential heparanase overexpression in this malignancy. The study demonstrates that advanced glycation end-products (AGE), typical components of the diabetic milieu, induce heparanase expression in PDAC, suggesting that the endoglycosidase contributes to sustaining the known bidirectional relationship between diabetes and pancreatic tumorigenesis. The authors propose that heparanase may exacerbate PDAC-associated diabetes, further contributing to tumor progression and therapy resistance, and suggest that heparanase targeting approaches disrupting this reciprocal causality may provide clinical benefit in PDAC (Goldberg et al.).

Further insights into the role of heparanase in tumor progression are provided by Cohen-Kaplan et al. The authors show that heparanase-mediated activation of Src results in the phosphorylation of catenins with the consequent destabilization of the E-cadherin/catenin complex and disruption of adherent junctions. Reduced integrity of epithelial sheets, a feature associated with advanced tumor stages, represents an additional effect whereby heparanase, through a mechanism likely independent of enzymatic activity, promotes cancer cell migration (Cohen-Kaplan et al.).

Overall, investigation of HSPGs and their endogenous modifying enzymes reveals a highly complex system affecting tumor growth and progression, and continues to inspire novel anticancer strategies exploiting pro- or anti-tumorigenic effects of the various system components. Articles in this Research Topic show the potential of this lively field of research to indicate novel tumor markers and treatments to be explored in specific disease settings.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Immunotherapeutic Targeting of GPC3 in Pediatric Solid Embryonal Tumors

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Glypican 3 (GPC3) is a heparan sulfate proteoglycan and cell surface oncofetal protein which is highly expressed on a variety of pediatric solid embryonal tumors including the majority of hepatoblastomas, Wilms tumors, rhabdoid tumors, certain germ cell tumor subtypes, and a minority of rhabdomyosarcomas. Via both its core protein and heparan sulfate side chains, GPC3 activates the canonical Wnt/β-catenin pathway, which is frequently overexpressed in these malignancies. Loss of function mutations in *GPC3* lead to Simpson-Golabi-Behmel Syndrome, an X-linked overgrowth condition with a predisposition to GPC3-expressing cancers including hepatoblastoma and Wilms tumor. There are several immunotherapeutic approaches to targeting GPC3, including vaccines, monoclonal antibodies, antibody-drug conjugates, bispecific antibodies, cytolytic T lymphocytes, and CAR T cells. These therapies offer a potentially novel means to target these pediatric solid embryonal tumors. A key pediatric-specific consideration of GPC3-targeted immunotherapeutics is that GPC3 can be physiologically expressed in normal tissues during the first year of life, particularly in the liver and kidney. In summary, this article reviews the current evidence for targeting childhood cancers with GPC3-directed immunotherapies.

Keywords: glypican 3, hepatoblastoma (HB), germ cell tumors (GCT), Wilms tumor (WT), rhabdoid tumor, rhabdomyosarcoma, neuroblastoma, immunotherapy

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INTRODUCTION

Glypican 3 (GPC3) is an oncofetal protein which is enriched on the surface of several pediatric solid embryonal tumors. This mini review evaluates the biological role of GPC3, synthesizes the published expression data in pediatric solid embryonal tumors, and describes the current immunotherapeutic approaches to target GPC3.

BIOLOGY

Glypicans are a highly conserved family of heparan sulfate proteolgycans which are attached to the plasma membrane via a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (1, 2). These surface proteins interact with growth factors to influence morphogenesis and are predominantly expressed during development (1, 2). Six glypicans (numbered 1–6) have been identified in humans and broadly are subdivided into two groups with GPC1, GPC2, GPC4, and GPC6 are the orthologs of *Dally* whereas GPC3 and GPC5 are the orthologs of *Dlp* in *Drosophila melanogaster* (1).

GPC3 is located on Chromosome Xq26 and encodes GPC3, also known as DGSX, GTR2-2, MXR7, OCI-5, SDYS, SGB, SGBS, and SGBS1 (2–4). During development, GPC3 is expressed in the placenta, fetal liver, fetal lung, and fetal kidney although it is absent or only minimally expressed in most adult tissues (5). This physiologic change may be mediated by suppression from DNA methylation within the *GPC3* promoter region (5–7).

GPC3 consists of an N-terminal domain that includes a secretory signal peptide as well as a GPI anchored C-terminal core protein containing two heparan sulfate chains (2–4). As with other glypicans, the GPC3 core protein and heparan sulfate side chains interact with a variety of regulatory proteins important in cell growth and differentiation, including Wnt, Hedgehog, and fibroblast growth factor (FGF) (8–12). In particular, GPC3 has been shown to interact with Wnts and binds directly to Frizzled, stimulating the formation of signaling complexes between these proteins which activates the canonical Wnt/β-catenin signaling pathway (8, 10). This signaling pathway is important for normal development of the kidney and liver, and is frequently aberrantly overexpressed in pediatric embryonal tumors (3, 8, 10, 13–17).

Simpson-Golabi-Behmel Syndrome (SGBS) is an X-linked overgrowth condition similar to the more common Beckwith-Wiedemann syndrome, and is associated with renal, hepatic, skeletal, and cardiac anomalies as well as predisposition to Wilms tumor, hepatoblastoma, and neuroblastoma (2, 18). SGBS is caused by constitutional microdeletions or truncating point mutations in *GPC3* which are predicted to result in a loss of function (2, 7, 18–21). Loss of GPC3 binding to insulin like growth factor 2 (IGF-2) was originally understood to cause this overgrowth phenotype but a series of subsequent papers demonstrates that this instead due, at least in part, to hyperactivation of Hedgehog signaling (20–24).

PEDIATRIC TUMORS

Pediatric malignancies derived from tissues that express GPC3 during development, such as the liver or kidney, frequently demonstrate upregulation of GPC3 which is likely important to both malignant transformation and tumorigenesis in these childhood cancers. GPC3 drives cell growth and inhibits differentiation via alterations in Wnt/β-catenin, Hedgehog, and FGF signaling which are often aberrantly expressed in pediatric embryonal tumors. In addition, alternative pathways not involved in physiologic GPC3 function, such as the Yap-Hippo pathway as has been shown in adult liver tumors, may also contribute to GPC3-mediated pediatric tumor development (25, 26). Finally, GPC3 has been reported to increase expression of the multi-drug resistance associated protein and therefore GPC3 in tumors may contribute to chemoresistance and treatment failure (27–29).

Abbreviations: CTL, Cytolytic T Lymphocytes; FGF, Fibroblast growth factor; GPC, Glypican; GPI, Glycosyl-phosphatidylinositol; HCC, Hepatocellular carcinoma; IGF, Insulin-like growth factor; IHC, Immunohistochemistry; NK, Natural killer; OCCC, Ovarian clear cell carcinomas; SGBS, Simpson-Golabi-Behmel Syndrome.

It is not fully understood how these childhood cancers are able to re-induce GPC3 expression. A study of the *GPC3* promoter methylation in primary pediatric embryonal tumors revealed gain of methylation mainly in boys with Wilms tumor and loss of methylation exclusively in girls with neuroblastoma (6). Increased tumor GPC3 expression was more commonly reported in a study of women than men with hepatocellular carcinoma (HCC), the most common adult liver tumor, although this has not been reproduced in subsequent studies (5). Thus, regulation of this X-linked gene may be not only age and tissue-specific but also gender-dependent and there are likely multiple means by which GPC3 becomes aberrantly deregulated in cancer. Nevertheless, across multiple studies, the extent of immunohistochemical (IHC) expression of GPC3 is relatively consistent for any given histology of embryonal tumor (**Figure 1**), each of which is to be reviewed in detail below.

Hepatoblastoma

There are a variety of studies that demonstrate that GPC3 is nearly universally expressed on most hepatoblastomas although may be absent in less typical subtypes (e.g., teratoid) or portions of hepatoblastoma with mesenchymal differentiation (30–36). GPC3 was the second most highly transcriptionally overexpressed gene in a study of 48 hepatoblastoma tumors compared to normal liver (37). Although highly expressed, multiple studies have found that soluble GPC3 is an inferior serum biomarker of hepatoblastoma response compared with alpha fetoprotein, the current standard of care (37, 38). Combining the results from 5 studies evaluating GPC3 expression via IHC in hepatoblastoma found that 131/135 (97%) cases demonstrate GPC3 expression, as shown in **Figure 1** (31–35).

Germ Cell Tumors

Several studies of extragonadal germ cell tumors demonstrate that yolk sac tumors and choriocarcionomas virtually always express GPC3 via IHC (**Figure 1**) (35, 39–41). In fact, GPC3 expression has been used to distinguish ovarian germ cell tumors from ovarian carcinomas (39). Other germ cell tumors, such as teratomas, embryonal carcinomas, and germinomas rarely express GPC3 (40, 41).

Wilms Tumors

Elevated transcriptional and proteomic expression of GPC3 is evident in a significant portion of Wilms tumors, as compared with adult kidney tumors and normal kidney tissue (36, 42, 43). Combining the results from 3 studies evaluating GPC3 expression in Wilms tumor revealed that 50/87 (58%) exhibit GPC3 expression, as shown in **Figure 1** (34, 35, 43). In addition to constitutional mutations seen in patients with SGBS, somatic tumor mutations in GPC3 have even been identified in some cases of Wilms tumors (44).

Rhabdoid Tumors

A series of 3 studies of extracranial malignant rhabdoid tumors demonstrate that 22/34 (65%) of these rare and highly aggressive tumors express GPC3 (34, 45, 46). Interestingly, other

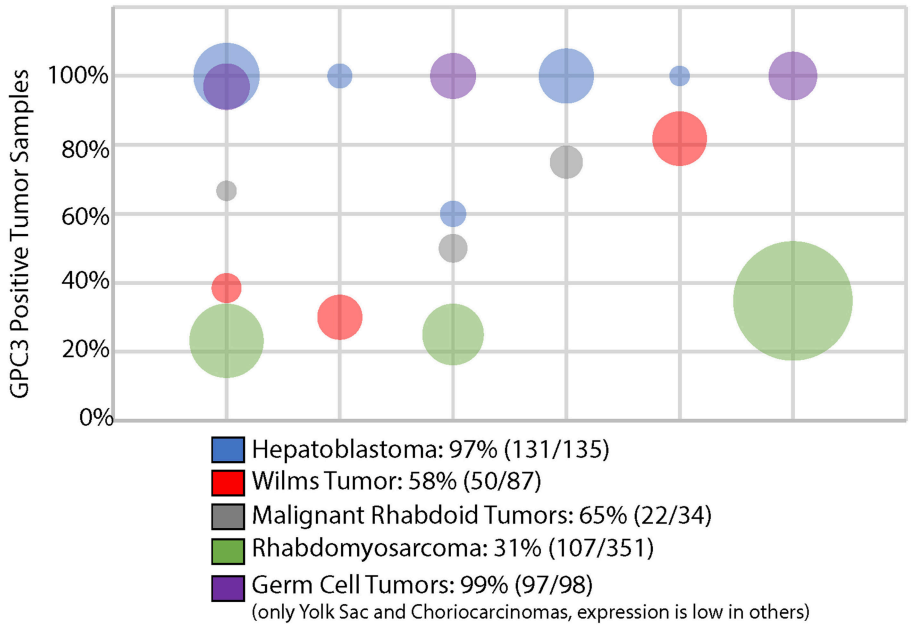

FIGURE 1 | GPC3 immunohistochemistry in pediatric solid embryonal tumors. Bubble area is proportionate to the number of tumors evaluated in a particular study.

extrarenal INI1 negative solid tumors except for undifferentiated sarcomas rarely express GPC3 (45). Given the challenging diagnostic overlap between some of these INI1 negative tumors, particularly extrarenal malignant rhabdoid tumor and epithelioid sarcoma, GPC3 may not only be a reasonable therapeutic target but may be helpful in improving diagnostic accuracy (45).

Rhabdomyosarcomas

A significant minority of rhabdomyosarcomas express GPC3, specifically 107/351 (31%) cases were positive in 3 different studies including both embryonal and alveolar subtypes (11, 34, 35, 47). Other pediatric sarcomas often do not express GPC3 (11, 47). Of all the glypicans, GPC3 exhibits the greatest homology with glypican 5 (GPC5), which is located on 13q32, a region of frequent genomic amplification in rhabdomyosarcomas and specifically associated with the PAX7-FOXO1 fusion (2, 11). Specifically in rhabdomyosarcomas, GPC5 has been specifically shown to potently activate Hedgehog signaling, which may be a result of its increased numbers of highly sulfated glycosaminoglan side chains compared with GPC3 (11, 48).

Neuroblastomas

There is mixed evidence regarding the role of GPC3 in neuroblastoma, with some studies showing increased expression in patients with 4S disease but most revealing absent expression of GPC3 in nearly all cases (34, 35, 42, 49, 50). The related glypican 2 (GPC2), however, has been shown to be an oncoprotein and immunotherapeutic target in high risk neuroblastoma (51, 52).

TREATMENTS

The development of GPC3-directed targeted therapies was stimulated by research into HCC where GPC3 was not only present, but also noted to be a prognostic biomarker in adults (4, 5, 12, 53, 54). These therapies have included vaccines, monoclonal antibodies, antibody-drug conjugates, bispecific antibodies, cytolytic T lymphocytes (CTL), and chimeric antigen receptors, which are described in more detail below.

Vaccines

From 2007 to 2009, a nonrandomized, open-label, phase I clinical trial with dose escalation of an HLA-A*24:02–restricted $GPC3_{298-306}$ peptide vaccine enrolled 33 Japanese adults with advanced HCC (UMIN 000001395) (12, 55). The vaccine elicited a GPC3-specific CTL response in 30 patients, notably with 1 partial response and 19 with stable disease 2 months after initiation of treatment (12, 55). Following this an open label, single arm, phase II study was performed in advanced HCC patients in Japan using the HLA-A*24:02–restricted $GPC3_{298-306}$ or HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine (UMIN 000002614) (12, 56). Although this study did not reach its primary endpoint, the 1 and 2 year event free survival was lower for the patients who underwent surgery alone as compared with those who received surgery plus vaccination (12, 56). This was statistically significant in a subgroup analysis of patients with GPC3 positive HCC (12, 56). These HLA-A*24:02

HLA-A2 GPC3-directed peptide vaccines were also used to treat Japanese patients with chemoresistant ovarian clear cell carcinoma (OCCC) (UMIN 000003696) (12, 57). This vaccine elicited a GPC3-specific CTL response in 15 of 24 patients who had peripheral blood mononuclear cells collected 3 times or more and 3 patients demonstrated a partial response (12, 57). Finally, a pediatric phase I study using these GPC3 directed vaccines was conducted in Japan and was found to be safe with a 2 month disease control rate of 66% (UMIN 000006357) (12, 58). A GPC3-specific CTL response was identified in 39% of patients in this study, the majority of whom were in remission and were diagnosed with hepatoblastoma (12, 58). To date, this is the only completed GPC3-directed immunotherapeutic clinical trial in pediatrics, as shown in **Table 1**.

Monoclonal Antibodies

Codrituzumab (RO5137382; RG7686; GC33) is a recombinant humanized antibody targeting GPC3 which interacts with CD16/FcγRIIIa on natural killer (NK) cells to cause antibody-dependent cytotoxicity in a GPC3-dependent manner (59–63). This drug has been studied in a series of 4 clinical trials in adults with HCC. For the first-in-man study in the US, 20 patients with advanced HCC were enrolled on a dose-escalation study of codrituzumab and a maximum tolerated dose was not reached as there were no dose limiting toxicities up to the highest planned dose level of 20 mg/kg weekly (61). Time to progression was statistically significantly higher in those HCC patients with higher GPC3 expression (61). A subsequent Japanese phase I study in advanced HCC patients revealed that 7/13 (54%) patients had stable disease, 3 of whom had prolonged (>5 month) disease stabilization (JapicCTI-101255) (62). In a phase Ib study in combination with sorafenib (NCT00976170), codrituzumab was not found to provide clinical benefit although this study demonstrated that ^{124}I radiolabeled codrituzumab was useful to monitor antibody uptake in the tumor and persistence of GPC3 expression after treatment (60). In a randomized placebo controlled phase II study (NCT01507168), codrituzumab similarly did not show a clinical benefit in advanced HCC patients, however combined elevation of tumor GPC3 and CD16/FcγRIIIa on NK cells correlated with survival (63–65). In HCC, expression of GPC3 has been shown to be a poor prognostic feature (66). Thus, even in these highest risk HCC patients with GPC3 expression, codrituzumab may provide clinical benefit, although monotherapy alone appears to be inadequate for HCC. Given the effectiveness of checkpoint inhibition with HCC, a combination of codrituzumab with the PD-L1 inhibitor atezolizumab is currently being evaluated in a Japanese phase I study of adult HCC patients (JapicCTI-163325). To date, codrituzumab is the only GPC3-directed immunotherapy to have a completed a clinical trial in the United States, as shown in **Table 1**.

The Ho Lab at the National Cancer Institute (Washington, DC, USA) has generated several additional GPC3-directed antibodies which have been extensively studied preclinically, including HN3, YP7, and HS20. HN3 is a GPC3-directed antibody that recognizes a cryptic Wnt binding domain and causes cell cycle arrest in HCC models via inactivation of Yap signaling (67). YP7 is another high affinity monoclonal antibody directed to the cell surface bound GPC3 and exhibited significant growth inhibition in HCC xenografts (68). HS20 is a human monoclonal antibody that recognizes the interaction site between the C-terminal GPC3 core fragment and heparan sulfate side chains in order to disrupt their interactions with Wnt (13, 69). This antibody was found to be an effective inhibitor of Wnt/β-catenin signaling *in vitro,* effectively inhibited HCC xenograft growth *in vivo*, and further was shown to impair cell migration and motility (13, 70).

Antibody-Drug Conjugates

Since GPC3 is efficiently internalized, it also is a good candidate for conjugation of antibodies to toxins (71). As a result, HN3 and YP7 were conjugated to the Pseudomonas endotoxin A and shown to be effective at reducing growth of xenografts *in vivo*, although notably the HN3-based drug conjugate, which is able to interfere with Wnt/β-catenin signaling, was more effective preclinically (71). There was significant *in vivo* toxicity so key immunogenic epitopes were removed from this antibody-drug conjugate, termed HN3-mPE24, in order to make it clinically viable (72).

Bispecific Antibodies

ERY974 is a bispecific antibody which targets both GPC3 (it was notably generated from codrituzumab) and CD3 and demonstrates *in vivo* antitumor efficacy against several GPC3 positive tumors (73). Intriguingly, ERY974 was effective even against tumors with nonimmunogenic features, by causing inflammation in the local tumor microenvironment (73). This is an important observation as even tumors which are not traditionally understood to be immunologically targetable on the basis of increased neoantigen expression could potentially be treated using this approach. More recently, Sano and colleagues presented results of a follow-up study which demonstrated synergy between ERY974 with Paclitaxel and Cisplatin (74). Given that Cisplatin is already an effective treatment modality for the majority of the GPC3 expressing pediatric solid embryonal tumors, this represents a promising opportunity for future combination studies. As shown in **Table 1**, an adult multicenter international phase I clinical trial of ERY974 is currently open in the United States and Europe (NCT02748837) and has planned expansion cohorts for stomach, esophageal, and other GPC3-expressing cancers.

Cytolytic T Lymphocytes

During the aforementioned peptide vaccination clinical trials, as well as a clinical study of HCC patients (UMIN 000005093), multiple peptide specific CTL clones were generated from peripheral blood and tumor tissue (12). These third party T cells are actively being developed for adoptive immune cell treatment of GPC3-positive tumors, as has been effectively utilized in the treatment of EBV associated post-transplantation lymphomas (12, 75, 76).

TABLE 1 | GPC3-targeted cancer immunotherapy trials.

Therapy name (Drug type)	Phase	Trial number	Eligibility	Status	Sponsor	Country
GPC3 Peptide Vaccine	I	UMIN 000001395	Adult HCC	Complete	National Cancer Center Hospital East	Japan
	II	UMIN 000002614	Adult HCC	Complete	National Cancer Center Hospital East	Japan
	II	UMIN 000003696	Adult OCCC	Complete	National Cancer Center Hospital East	Japan
	I	UMIN 000006357	**Pediatric** GPC3+ Tumors	Complete	National Cancer Center Hospital East	Japan
Codrituzumab (Monoclonal Antibody)	I	NCT 00746317	Adult HCC	Complete	Chugai Pharmaceutical	USA
	I	JapicCTI 101255	Adult HCC	Complete	Chugai Pharmaceutical	Japan
	I*	NCT 00976170	Adult HCC	Complete	Chugai Pharmaceutical	USA
	II	NCT 01507168	Adult HCC	Complete	Hoffman-La Roche	USA
	I**	JapicCTI 163325	Adult HCC	Open	Chugai Pharmaceutical	Japan
ERY974 (Bispecific Antibody)	I	NCT 02748837	Adult HCC	Open	Chugai Pharmaceutical	Multi-National
GAP T cells (CAR T Cell)	I	NCT 02932956	**Pediatric** GPC3+ Liver Tumors	Open	Baylor College of Medicine	USA
GLYCAR T cells (CAR T Cell)	I	NCT 02905188	Adult HCC	Open	Baylor College of Medicine	USA

**Combination with Sorafenib.*
***Combination with Atezolizumab.*
Bold text refers to pediatric studies.

Chimeric Antigen Receptors

The Heczey Lab at Baylor College of Medicine (Houston, TX, United States) has generated several GPC3-targeted chimeric antigen receptor (CAR) constructs (77). Notably all of these GPC3/CARs rendered T cells highly cytotoxic to GPC3-positive HCC, hepatoblastoma, and malignant rhabdoid tumor cell lines *in vitro* as well as HCC and malignant rhabdoid tumors *in vivo* (77). The GPC3 directed CAR with the 4-1BB Zeta chain was the most effective at inducing T cell expansion and proliferation (77). As a result, two clinical trials are currently in development, GLYCAR T cells (NCT02905188) for adults with HCC and GAP T cells (NCT02932956) for children aged 1–21 with GPC3 positive liver tumors (**Table 1**).

CHALLENGES

Although GPC3 is expressed in a wide variety of pediatric solid tumors, it is also expressed physiologically in infants, predominantly in the liver and kidney, with detectable serum levels during the first year of life (35). Thus, GPC3 targeted therapies could cause significant toxicity not seen in adults thus far due to persistent physiologic expression of GPC3 in the liver and kidney. If indeed clinical trials in pediatrics reveal immunogenic targeting of normal tissues, strategies to limit toxicity will need to be employed, such as limiting age to children >1 year of age as is being done in the GAP T cell study (NCT02932956). Given the generalized expression of GPC3 in the fetus and placenta, GPC3 based immunotherapies are likely to be teratogenic. Care must be made when counseling and treating women of childbearing age with GPC3-based immunotherapies. Finally, immunotherapies targeting these cancers need to be designed such that they preferntially target the core C-terminal GPC3 protein, its heparan sulfate side chains, or their interactome rather than the soluble N-terminal GPC3. In fact, soluble GPC3 expression may be useful as a biomarker of response to GPC3 therapies.

CONCLUSIONS

The heparan sulfate proteoglycan GPC3 is an attractive target for drug development as it is highly upregulated in HCC and several pediatric solid embryonal tumors and is responsible for driving key growth and developmental pathways which are currently not effectively targeted using our existing therapies (2, 12, 78). At this point, there is very limited clinical experience with GPC3-directed immunotherapeutics in pediatric oncology: A GPC3-directed vaccine study was conducted in Japan for children with solid tumors expressing GPC3 (UMIN 000006357) and in December 2018, the GAP CAR T cell study (NCT02932956) opened for children and young adults with GPC3-expressing liver tumors (12, 58). Vaccines, monoclonal antibodies, antibody-drug conjugates, bispecific antibodies, CTLs, and CAR T cell based therapies are all emerging treatment options which may provide enhanced ability to target GPC3 in pediatric solid embryonal tumors. As ongoing clinical trials in adults demonstrate which of these GPC3-based modalities are safe and beneficial, it is imperative that we rigorously evaluate the role of these

potentially life-saving therapies in children and adolescents with GPC3-driven tumors.

AUTHOR CONTRIBUTIONS

MO generated the initial draft of the manuscript. All authors reviewed the manuscript for content and accuracy and are responsible for its content.

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Heparanase Promotes Tumor Growth and Liver Metastasis of Colorectal Cancer Cells by Activating the p38/MMP1 Axis

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Heparanase (HPSE), the only known mammalian endoglycosidase responsible for heparan sulfate cleavage, is a multi-faceted protein affecting multiple malignant behaviors in cancer cells. In this study, we examined the expression of HPSE in different colorectal cancer (CRC) cell lines. Gene manipulation was applied to reveal the effect of HPSE on proliferation, invasion, and metastasis of CRC. Knockdown of HPSE resulted in decreased cell proliferation *in vitro*, whereas overexpression of HPSE resulted in the opposite phenomenon. Consistently, *in vivo* data showed that knockdown of HPSE suppressed tumor growth of CRC. Furthermore, knockdown of HPSE inhibited invasion and liver metastasis *in vitro* and *in vivo*. RNA-sequencing analysis was performed upon knockdown of HPSE, and several pathways were identified that are closely associated with invasion and metastasis. In addition, HPSE is positively correlated with MMP1 expression in CRC, and HPSE regulates MMP1 expression via p38 MAPK signaling pathway. In conclusion, our data demonstrate that HPSE knockdown attenuated tumor growth and liver metastasis in CRC, implying that HPSE might serve as a potential therapeutic target in the treatment of CRC.

Keywords: colorectal cancer, heparanase, liver metastasis, MMP1, extracellular matrix

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INTRODUCTION

Colorectal cancer (CRC) is the third most common diagnosed cancer and the fourth leading cause of cancer-related mortality worldwide. Moreover, tumor invasion and metastasis are the main causes of mortality in CRC patients (1, 2). The liver is the most common site of CRC metastasis and more than 50% of CRC patients will develop metastatic liver disease (3, 4). Therefore, identification of the mechanisms involved in the invasion and metastasis of CRC is urgently needed.

The first process in the invasion and metastasis of tumor cells is the breakage of the tissue barrier formed by the basement membrane and extracellular matrix (ECM). Heparanase (HPSE), the only recognized mammalian endo-β-D-glucuronidase, is responsible for the cleavage of heparan sulfate (HS) chains of heparan sulfate proteoglycans (HSPGs) in the ECM as well as on the tumor cell surface (5). HPSE is first synthesized as a latent ∼65 kDa precursor protein and then post-translationally cleaved into ∼8 and ∼50 kDa subunits that non-covalently associate to

form the active HPSE heterodimer (6). HSPGs are important components of the ECM, and the activity of HPSE could lead to the degradation of the ECM, which facilitates tumor cell invasion (7). In addition, HS serves as a storage depot for many cytokines, including VEGF, bFGF and HGF (8, 9). Cleavage of HS by HPSE leads to the release of bioactive cytokines, which promotes tumor growth and angiogenesis. The upregulation of HPSE has been implicated in many types of cancers, including CRC (7, 10–13). Both HPSE mRNA and protein are practically undetectable in morphologically normal colon epithelium, but are induced during colon carcinogenesis (14–16). The presence of HPSE in CRC correlates with higher TNM stage, higher vascular infiltration, and higher lymph vessel infiltration (15). In addition, HPSE expression is frequently detected in the invasive front of CRC (15). Furthermore, the 5-year survival rate is higher for patients with negative HPSE expression, and significant correlations were reported between HPSE expression and liver metastasis (10). Importantly, HPSE-transfected RPMI 4788 CRC cells showed increased invasiveness in invasion chamber assays, and the HPSE inhibitor SF-4 suppressed the invasion of RPMI 4788 cells (15). Although the clinical significance of HPSE is well documented, the mechanisms of HPSE-mediated CRC invasion and metastasis need to be further explored.

In this study, CRISPR/Cas9 technology was used to manipulate HPSE expression in CRC cells. We showed that knockdown of HPSE suppresses CRC cell proliferation, invasion, and liver metastasis. RNA-sequencing revealed that genes and pathways involved in remodeling of ECM were attenuated upon HPSE knockdown. These finding suggest that HPSE might be an attractive target for the treatment of CRC.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human colorectal cancer cell lines SW480, SW620, LoVo, HT-29, and HCT116, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco). All cells were cultured in a humidified 5% CO_2 incubator at 37°C.

Lentiviral Constructs and Cell Infection

HPSE overexpression was induced with a lentiviral vector system, as previously described (13). Briefly, SW620 cells were infected with HPSE lentiviral activation particles or control lentiviral activation particles (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The lentiviral activation particles contain the following SAM activation elements: a deactivated Cas9 nuclease (D10A and N863) fused to the transactivation domain VP64, and a blasticidin resistance gene; an MS2-p65-HSF1 fusion protein, and a hygromycin resistance gene; a target-specific 20nt guide RNA, and a puromycin resistance gene. Stable infected cells were selected with 2 μg/mL puromycin, 400 μg/mL hygromycin B and 5 μg/mL blasticidin S HCl. For convenience, these two cell lines are referred to as SW620-HPSE and SW620-Con, respectively.

TABLE 1 | The sgRNAs sequences.

Number	sgRNA (5–3′)
NC	CGCTTCCGCGGCCCGTTCAA
KD1	ACGGTTGGAATGGCCCTACC
KD2	TTCTCCAAAGCTTCGTACCT

For construction of the HPSE knockdown cell line, SW480 cells were infected with Lenti-CAS9-puro (GeneChem Co. Ltd. Shanghai, China) and selected with 2 μg/mL puromycin. Then, cells stably expressing SW480-Cas9 were infected with two lentivirus-sgRNA-EGFP vectors targeting HPSE and a control lentivirus-sgRNA-EGFP, respectively. The sgRNA sequences are listed in **Table 1**. Exon 4 and Exon 3 of HPSE gene were targeted by KD1 and KD2, respectively. Stable HPSE knockdown cells are referred to as SW480-KD1, SW480-KD2, whereas control cells were named SW480-NC.

RNA Extraction and Real-Time Reverse Transcription-PCR

Total cellular RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified using a UV spectrophotometer. RNA was then reverse transcribed to cDNA using PrimerScript^{TM} RT master mix (Takara, Dalian, China). mRNA expression was examined by real-time reverse transcription PCR (RT-PCR) using SYBR Green Mix with a CFX96 real-time PCR system (Bio-rad, Richmond, CA, USA). Each reaction was performed in triplicate. The results were normalized to human β-actin mRNA expression. The primers used in this study are listed in **Table S1**.

Western Blot Analysis

Proteins were extracted from samples using radio immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) according to the manufacture's protocol. After gel electrophoresis, PVDF membranes were blocked in 5% milk/PBS-T for 2 h followed by overnight incubation at 4°C with antibodies against HPSE (Proteintech, Chicago, IL, USA; 1:1000, 66226-1-Ig), phosphor-p38 (Cell Signaling, Beverly, MA, USA; 1:2000, 4511), p38 (Cell Signaling; 1:2000, 8690), MMP1 (Proteintech; 1:1000, 10371-2-AP), PCOLCE (OriGene Tech, Rockville, MD, USA; 0.5 μg/mL, TA337676), MMP10 (Abcam, Cambridge, MA, USA; 1:1000, ab199688), CEACAM6 (Abcam; 1:500, ab78029) and β-actin (Sigma- Aldrich, St Louis, MO, USA; 1:5000, A5316). After washes and incubation with respective horseradish peroxidase-conjugated secondary antibodies for 2 h, protein bands were visualized using the SuperSignal West Pico maximum sensitivity substrate (Pierce, Rockford, IL, USA).

Immunofluorescence Staining

Cells were seeded into 24-well plates containing glass coverslips on the bottom of the wells. Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with Triton X-100 for 10 min, and blocked with 2.5% bovine serum albumin for 1 h. Cells were then incubated with the anti-HPSE antibody

(Proteintech; 1:200, 66226-1-Ig) for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated secondary antibody for 1 h. The cell nucleus was stained with DAPI for 5 min. Images were captured with an inverted fluorescence microscope (PerkinElmer, Norwalk, CT, USA).

Cell Proliferation Assay

Cell proliferation was determined with the CCK-8 assay. Cells were incubated in 10% CCK-8 (Beyotime), which was diluted in normal culture medium for 1 h at 37°C. The absorbance was measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Invasion Assays

Invasion assays were performed using 8 μm Transwell chambers coated with Matrigel (BD Biosciences, Bedford, MA, USA). Briefly, cells were suspended in 100 μL serum-free medium and seeded into the upper chamber. The lower chamber was filled with 600 μL DMEM medium supplemented with 10% FCS. After 24 h of incubation, invaded cells were stained with 0.5% crystal violet (Beyotime) and examined by bright field microscopy (Leica, Wetzlar, Germany).

Animal Experiments

For tumorigenicity assay, 1×10^6 cells were injected subcutaneously into the back of BALB/c nude mice. After 3 weeks, mice were sacrificed; tumor xenografts were harvested, weighted, and fixed in formalin.

For the *in vivo* liver metastasis assay, SW480 cells (2×10^6) were suspended in PBS and then injected into the spleen of 6–8 weeks old male BALB/c nude mice. Five weeks after intrasplenic injection, mice were sacrificed, and spleen and livers specimens were fixed in formalin. Sections (5-μm thickness) of the liver were made at 10 different layers to cover the entire organ and stained with hematoxylin and eosin (H&E). Metastatic foci were counted under microscopy in a double-blinded manner. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee.

Immunohistochemical Analysis and Scoring

Immunohistochemistry was performed as previously described. Briefly, following deparaffinization and rehydration, the sections were boiled in 10 mmol/L citrate buffer (pH 6.0) for 15 min in a microwave oven. The sections were then incubated with anti-HPSE (Proteintech; 1:100, 66226-1-Ig) or anti-Ki67 (MXB Biotech, Fujian, China; 1:100, MAB-0672) antibodies overnight at 4°C. Sections were washed for 2 h in TBST and then incubated with secondary antibody (DAKO, Carpinteria, CA, USA; P0447) at a dilution of 1:100 in TBST. Finally, the sections were visualized using diaminobenzidine solution (DAKO). The results were verified by two pathologists independently.

RNA Sequencing and Data Analysis

Three independent experiments were performed for each group to obtain biological replicates. RNA was extracted and sequenced by CapitalBio Corporation (Beijing, China). The NEB Next Ultra RNA Library Prep Kit for Illumina (NEB, Beverly, MA, USA) was used to construct libraries for sequencing. The final libraries were quantified using the KAPA Library Quantification kit (KAPA Biosystems, South Africa) and Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA); libraries were then sequenced on a Hiseq 2500 platform (Illumina, San Diego, USA).

Differentially expressed genes (DEGs) were assessed using Cuffdiff, with a false discovery rate correction for multiple testing. DEGs were considered significant when the log2 signal ratio was ≥ 1.0 or ≤ -1.0 with a p-value < 0.05 between comparisons. By searching the ENSEMBL, NCBI, Uniprot, GO, and KEGG databases, BLAST (Basic Local Alignment Search Tool) alignment was performed to determine the functional annotation of DEGs. The best matches were selected to annotate the DEGs. Then, these DEGs were grouped and analyzed using Gene Ontology (GO) enrichment analysis. And, these DEGs were grouped into gene pathways using the pathway enrichment analysis with the following databases: KEGG, BioCyc, Reactome, and Panther. Finally, the protein-protein interaction analysis was conducted using STRING database and visualized using Cytoscape software (Cytoscape Consortium, San Diego, CA, USA).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 5.0 (Graphpad, San Diego, CA, USA) using Student's t-test. Data are presented as the mean ± standard deviation except for where noted. $P < 0.05$ was considered statistically significant.

RESULTS

HPSE Expression in CRC Cell Lines

The expression of HPSE at the mRNA and protein level was evaluated in five CRC cell lines by qRT-PCR and western blotting, respectively. As shown in **Figures 1A,B**, SW480, and HCT116 cells express higher levels of HPSE mRNA and protein compared to that in other cells. The lowest expression of HPSE was found in SW620 cells. Immunofluorescence staining revealed that HPSE is located primarily in the cytoplasm of CRC cells (**Figure 1C**). Since SW480 and SW620 cells were isolated from the same patient, they were selected as the "host" cell to evaluate the functional properties of HPSE in colorectal cancer cells.

HPSE Promotes Proliferation of CRC Cells *in vitro*

The effect of HPSE on proliferation was reported in other malignancies, but it remains to be investigated in CRC cells. To this end, two gRNAs targeting different regions of the HPSE gene were designed and we transfected the gRNA-Cas9 expression vectors into SW480 cells to generate HPSE knockdown cells (**Figure 2A**), while SW620 cells were stably transfected with HPSE-expressing particles (**Figures 2B,C**). Using the CCK-8 assay, we found that HPSE overexpression resulted in significantly increased cell proliferation; in contrast, HPSE knockdown markedly decreased the proliferation of CRC cells (**Figures 2D,E**).

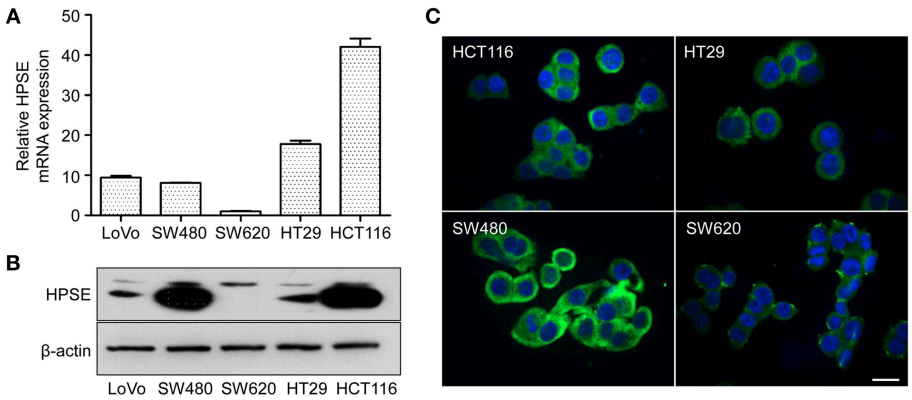

FIGURE 1 | HPSE expression in CRC cells. **(A)** Quantification of HPSE mRNA expression in different CRC cells. The expression of HPSE was normalized to β-actin. Results are shown as the fold change of CRC cells relative to SW620 cells. Results are representative of three independent experiments and are presented as the mean ± SEM. **(B)** Western blot analysis of HPSE expression in whole-cell lysates of CRC cells. SW480 and HCT116 cells exhibited higher expression of HPSE compared to the other cell lines. **(C)** Immunofluorescence staining demonstrated that HPSE was mainly localized in the cytoplasm of CRC cells. Green indicates HPSE and blue indicates DAPI. Scale bar, 20 μm.

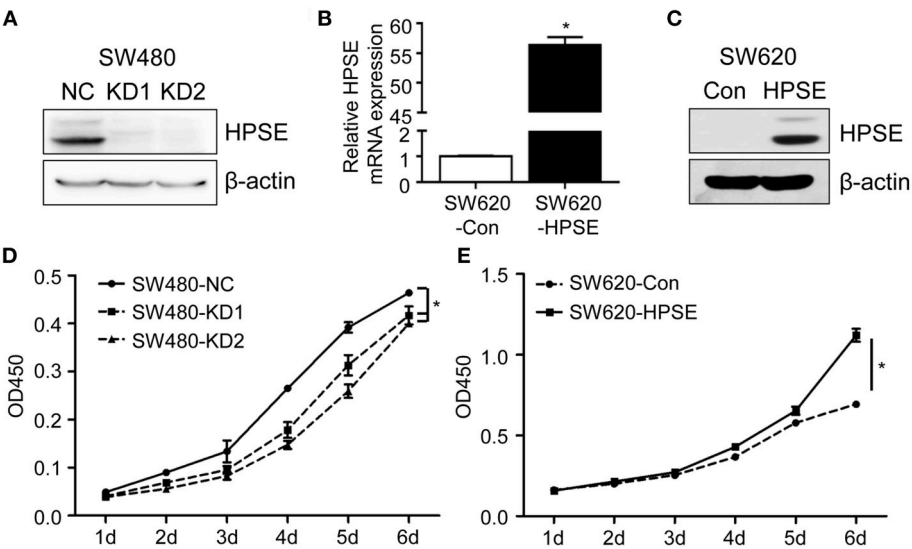

FIGURE 2 | HPSE promotes the proliferation of CRC cells *in vitro*. **(A)** Crispr-Cas9 technology was used to knockdown HPSE expression in SW480 cells and the expression of HPSE was determined by western blotting. **(B,C)** SW620 cells were infected HPSE lentiviral activation particles and HPSE expression was determined by **(B)** qRT-PCR and **(C)** western blotting. The proliferation of **(D)** SW480 cells and **(E)** SW620 cells was determined by the CCK-8 assay ($n = 3$). The OD450 value was assessed at 1, 2, 3, 4, 5, and 6 days respectively. *$p < 0.001$.

HPSE Accelerates Tumor Growth of CRC Cells *in vivo*

To evaluate the role of HPSE in the proliferation of CRC *in vivo*, CRC cells were subcutaneously injected into the flank region of BALB/c nude mice and xenografts were harvested at 3 weeks after transplantation. As shown in **Figure 3A**, the tumor weight of the SW480 HPSE-knockdown group was much smaller than that in the control group (0.114 ± 0.038 g, 0.127 ± 0.054 g vs. 0.326 ± 0.065 g, $p < 0.001$). In contrast, tumors formed by SW620-HPSE cells were larger than that in SW620-Con cells (0.328 ± 0.202 g vs. 0.1376 ± 0.037 g) (**Figure 3B**). As Ki67 is frequently used to assess proliferation in human cancer cells, immunohistochemistry analysis was performed to determine Ki67 and HPSE expression in tumors derived from HPSE overexpression and knockdown cells. Representative pictures of Ki67 staining are shown in **Figures 3C,D**. Compared with SW480-NC group, Ki-67 quantification revealed a significant reduction of tumor cell proliferation in HPSE-knockdown group (59.14 ± 3.43% vs. 35.13 ± 3.14%, 32.50 ± 4.90%, $p < 0.001$) (**Figure 3E**). Conversely, higher tumor cell proliferation was observed in SW620-HPSE group compared to SW620-Con group (74.16 ± 5.74% vs. 48.59 ± 5.00, $p < 0.001$) (**Figure 3F**). These

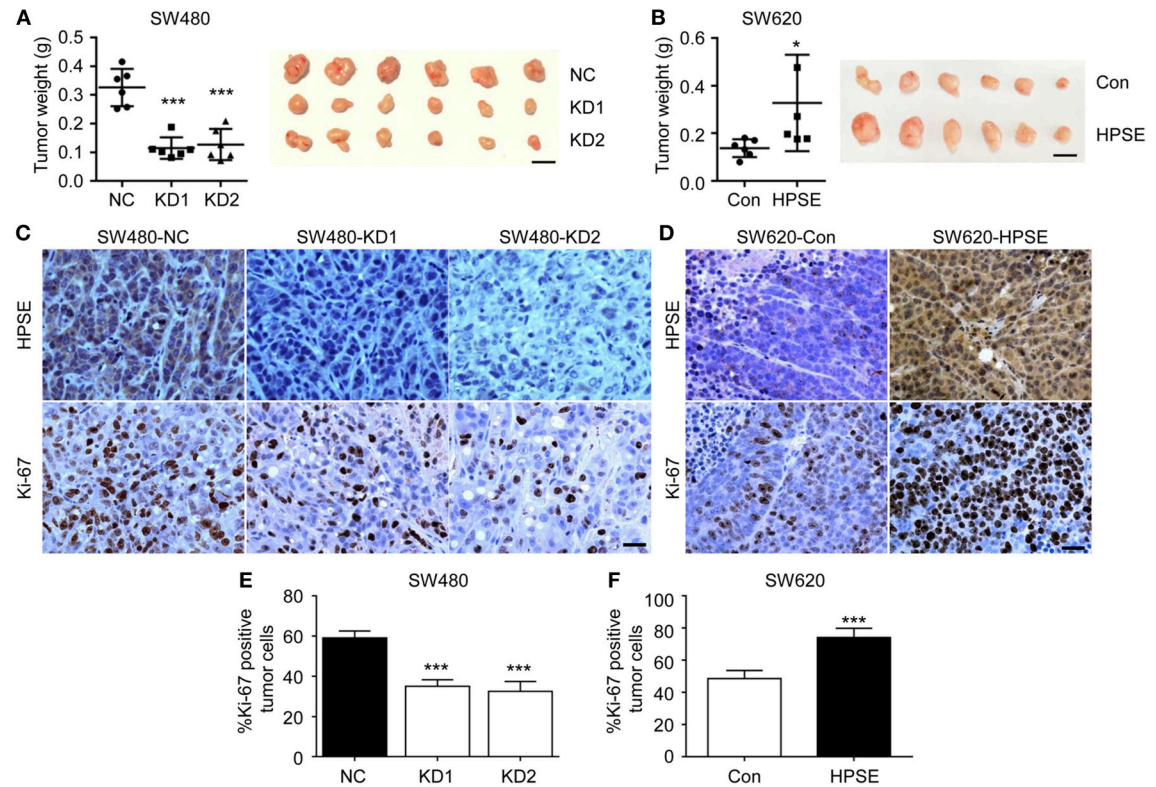

FIGURE 3 | HPSE promotes the proliferation of CRC cells *in vivo*. Cells were injected subcutaneously into the back of BALB/c nude mice. Mice were sacrificed at 3 weeks after transplantation. The xenografts were excised and weighted. **(A)** Knockdown of HPSE expression inhibited tumorigenicity of SW480 cells in BALB/c nude mice ($n = 6$). The weight of tumors originating from SW480-NC is larger than that arising from SW480-KD1 and SW480-KD2 cells (left panel). A representative photograph of tumor size is shown (right panel). **(B)** Overexpression of HPSE in SW620 cells promoted the growth of mouse xenograft tumors ($n = 6$). Scale bar, 1 cm. Immunohistochemistry analysis of HPSE and Ki-67 expression in xenografts originating from **(C)** SW480 and **(D)** SW620 cells. Scale bar, 100 μm. Quantification of tumor cell proliferation in **(E)** SW480 and **(F)** SW620 xenografts using Ki-67 staining ($n = 6$). Data are expressed as Ki-67 positive tumor cells as percentage of total tumor cells. $^{*}p < 0.05$, $^{***}p < 0.001$.

data indicate that HPSE promotes the proliferation of CRC cells *in vivo*.

Knockdown of HPSE Suppresses the Invasion and Metastasis of CRC Cells

To explore the effect of HPSE on the invasion of CRC cells, an invasion assay was used to determine the invasiveness of SW480 cells after HPSE knockdown. After 24 h, the cells on the lower surface of the chamber were fixed, stained, and examined under a microscope. Knockdown of HPSE was found to significantly suppress the invasive ability of SW480 cells (**Figures 4A,B**). To further investigate the pro-metastatic activity of HPSE, SW480 cells were injected into the spleen of 6- week-old male BALB/c nude mice. After 5 weeks, the mice were sacrificed, and their livers were fixed. Metastatic foci in the livers were counted. Our results showed that liver metastasis was significantly inhibited by HPSE knockdown (**Figures 4C,D**). Combining the two HPSE knockdown groups, metastasis was found in the livers of 50% (4/8) of the mice. In contrast, metastatic foci were found in all livers of the control group. These results suggest that HPSE is critical for tumor invasion and metastasis in CRC.

Identification of DEGs in HPSE-Knockdown SW480 Cells

To better understand the role of HPSE in CRC cell invasion and metastasis, we took advantage of next generation RNA sequencing technology to analyze mRNA transcriptome differences between SW480-KD1 and SW480-NC cells. The data have been deposited under GEO accession number GSE126504. We identified a total of 104 genes that were significantly upregulated (**Table S2**) and a total of 83 genes that were significantly downregulated (**Table S3**) in SW480-KD1. To obtain a global view of these DEGs, hierarchical clustering was constructed (**Figure 5A**). Additionally, the degree of expression change of these DEGs between the two groups is shown as a volcano plot in **Figure 5B**. To further validate the reliability of the RNA-seq results, twelve DEGs were selected for qRT-PCR validation, including *PCOLCE*, *COL7A1*, *FUT3*, *CASP10*, *FBLN5*, *PTGS2*, *CXCL8*, *MMP13*, *MMP7*, *MMP10*, *MMP1*, and *CEACAM6* (**Figure 5C**). The qRT-PCR results are in accordance with RNA-seq data. Western blot analysis was also used to validate some DEGs, including MMP1, PCOLCE, MMP10, and CEACAM6 (**Figure 5D**). Except for CEACAM6, the protein

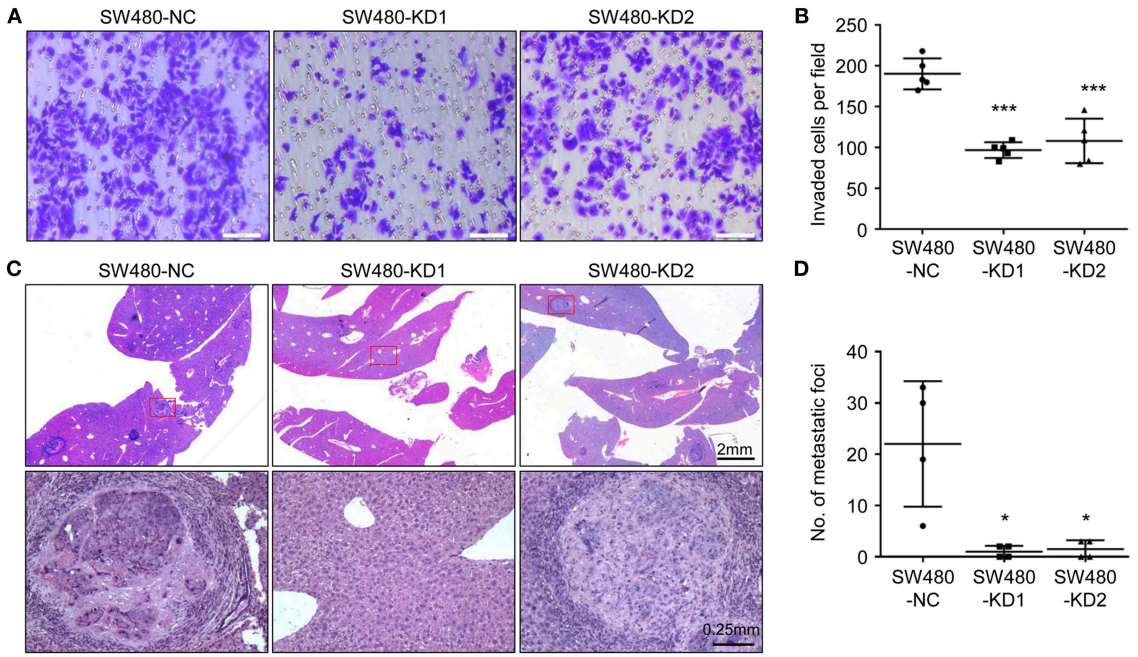

FIGURE 4 | Knockdown of HPSE inhibits CRC cells invasion *in vitro* and liver metastasis *in vivo*. **(A)** An invasion assay was performed in a Matrigel-coated transwell chamber; representative images of invasion assay are shown. **(B)** The invasive ability of SW480 cells was inhibited by HPSE knockdown ($n = 5$). **(C)** SW480 cells were injected into the spleens of BALB/c mice. After 6 weeks, the mice were sacrificed, and their livers were fixed. Microscopic liver metastases were detected by H&E staining. **(D)** The metastatic foci were counted ($n = 4$). HPSE knockdown in SW480 cells resulted in fewer metastatic foci in the liver. $^{*}p < 0.05$, $^{***}p < 0.001$.

levels of MMP1, PCOLCE, and MMP10 are in accordance with RNA-seq data.

To identify signaling pathways involved in HPSE-knockdown cells, we mapped the ENSEMBL, NCBI, Uniprot, GO, and KEGG databases; the top 20 enriched pathways are shown in **Figure 6A** and **Table S4**. DEGs were highly clustered in several pathways, such as "collagen degradation," "activation of matrix metalloproteinases." "Extracellular matrix organization" and "degradation of the extracellular matrix," suggesting that HPSE may perform its function through the regulation of genes involved in the remodeling of the extracellular matrix.

GO analysis was then performed to group genes with similar function and associations. The top 20 enriched GO terms are shown in **Figure 6B** and **Table S5**. In the cellular component category, the DEGs were mostly enriched in plasma membrane part. In the molecular function category, genes associated with serine-type endopeptidase inhibitor activity were enriched. Notably, in the biological process analysis, genes involved in extracellular matrix organization were significantly enriched, including *MMP1*, *MMP7*, *MMP10*, *MMP13*, *COLA71*, and *FBLN5*.

HPSE Is Positively Correlated With MMP1 in CRC

Knockdown of HPSE is accompanied by downregulation of *MMP1*, *MMP7*, *MMP10*, and *MMP13*, all of which are directly involved in the degradation of extracellular matrix and facilitate tumor cell invasion. We thus further analyzed the data from TCGA and showed that *HPSE* is positively correlated with the expression of *MMP1* in colon cancer ($r = 0.3476$, $p < 0.0001$) and rectal cancer ($r = 0.3428$, $p < 0.0001$) (**Figures 7A,B**), but not *MMP7*, *MMP10*, and *MMP13* (**Figure S1**).

It has been shown that MMP1 is regulated by the p38 MAPK signaling pathway in colorectal cancer (17). Our results also showed that knockdown of HPSE decreased the expression of MMP1 and the phosphorylation of p38. By contrast, HPSE overexpression led to increased levels of MMP1 and phosphorylation of p38, and the increase was abolished by the treatment of SB203580 (a p38 pathway inhibitor) (**Figure 7C**). In addition, Cytoscape software was used to analyze protein-protein interaction of these differentially expressed genes. As shown in **Figure 7D**, MAPK14 (p38) was most enriched with 26 proteins connected, of which MMP1 was one of the proteins having the highest score (score = 882) (**Table S6**). Collectively, these data indicate that HPSE might regulate MMP1 expression via the p38 MAPK signaling pathway.

DISCUSSION

The liver is the most common site of CRC metastasis because the majority of intestinal mesenteric drainage enters the hepatic portal venous system (18). High expression of HPSE has been reported in CRC and is correlated with poor prognosis and liver metastasis (10, 15). However, it is still not clear

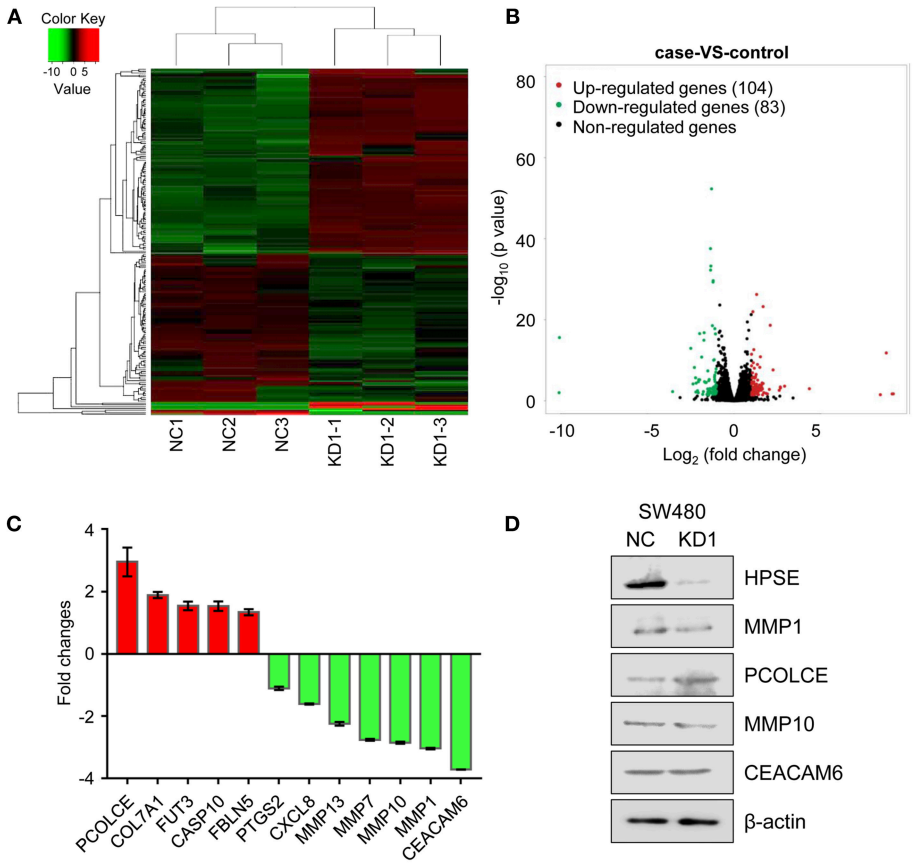

FIGURE 5 | Differentially expressed genes across all samples. **(A)** Hierarchical clustering of differentially expressed mRNA between SW480-KD1 and SW480-NC cells. The relative levels of differentially expressed genes are depicted on the color scale. Red indicates increased relative expression and green indicates decreased relative expression. **(B)** Distribution of the differentially expressed genes is shown as a volcano plot. The detected genes are presented using log2 (fold change) on the x-axis and -log10 (*p*-value) on the y-axis. **(C)** Twelve differentially expressed genes were selected and validated using qRT-PCR. Results are representative of three independent experiments and are presented as the mean ± SEM. **(D)** The protein levels of HPSE, MMP1, PCOLCE, MMP10, and CEACAM6 were also validated by Western blot analysis. β-actin was used as internal control.

whether HPSE is directly involved in CRC cell invasion and metastasis. Here, we demonstrated that the proliferation, invasion and liver metastasis of CRC cells are inhibited by HPSE knockdown.

HPSE is a highly versatile protein affecting multiple events in tumor progression, including cell adhesion, invasion and angiogenesis. Strategies targeting HPSE have also been developed, including neutralizing antibody, heparan sulfate mimetics, and siRNA (19). CRISPR-Cas9 is a new gene editing technique with high target-specificity, and there is great interest in evaluating its potential for human gene therapy (20). In this study, two gRNAs targeting HPSE were shown to efficiently knockdown HPSE expression (**Figure 2A**). Several groups have reported that inhibition of HPSE expression by small interference RNA resulted in decreased proliferation and invasion in other malignancies (21–23). Consistent with those results, we also demonstrate that knockdown of HPSE inhibits proliferation and invasion of SW480 cells. In contrast, overexpression of HPSE in SW620 cells resulted in increased proliferation (**Figures 2**,**3**). Furthermore, Doniner et al. also reported that overexpression of HPSE in HT29 CRC cells promote xenografts growth (16). Of particular interest, our data demonstrated that knockdown of HPSE attenuated CRC liver metastasis in a mouse model of liver metastasis (**Figure 4**). To the best of our knowledge, this is the first study to apply CRISPR-Cas9 technology to knockdown of HPSE expression and provide direct evidence of the role of HPSE in liver metastasis of CRC.

Since the mechanisms involved in HPSE-mediated invasion and metastasis have not yet been elucidated, we utilized RNA-seq technology to profile differentially expressed genes and pathways in HPSE-knockdown CRC cells. A total of 187 genes were identified, of which 104 genes were upregulated and 83 downregulated (**Figure 5**). Interestingly, several genes associated with cancer cells invasion were downregulated upon HPSE knockdown, including *MMP1*, *MMP7*, *MMP10*, *MMP13*, and *CEACAM6* (**Figure 5C**). Decreased MMP1 and MMP10 protein levels were also observed in HPSE knockdown cells, but not for

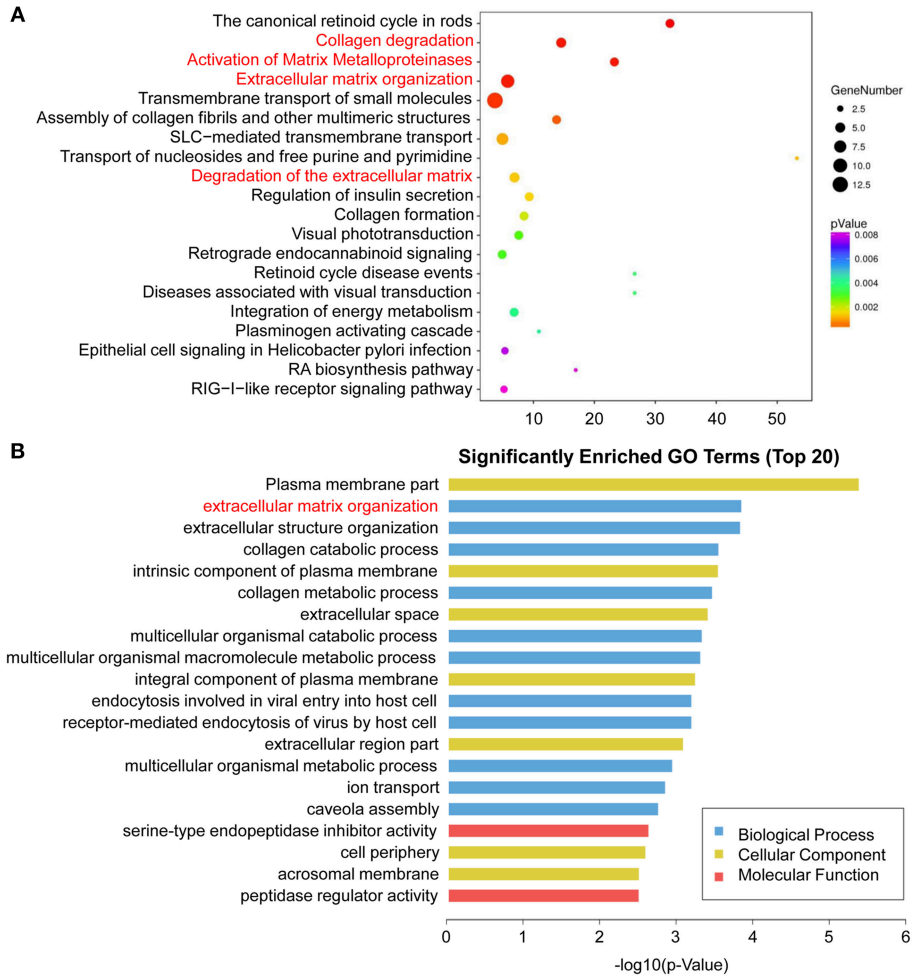

FIGURE 6 | Enriched pathway statistics and GO functional classification of the DEGs. **(A)** Scatter plot of enriched pathway statistics. These differentially expressed genes were grouped into gene pathways using the pathway enrichment analysis with the databases of KEGG, BioCyc, Reactome, and Panther. The color and size of the dots represent the range of the *p*-value and the number of DEGs mapped to the indicated pathways, respectively. Top 20 enriched pathways are shown. **(B)** Top 20 significantly enriched GO terms. The DEGs are summarized in three main categories: biological process (blue), molecular function (red), and cellular component (yellow). The x-axis indicates -log10 (*p*-value) and the y-axis indicates different GO terms.

CEACAM6 (**Figure 5D**). In addition, PCOLCE was upregulated in HPSE knockdown cells and PCOLCE might have an MMP inhibitory activity (24). Of note, we showed that *HPSE* is positively correlated with *MMP1* expression by analyzing TCGA database (**Figures 7A,B**). MMP1 is a collagenase that degrades ECM, especially type I, II and III collagens. In CRC, MMP1 expression correlates with advanced stage and poor prognosis, and CRC invasion and migration correlated with increased MMP1 expression (25). Previously, Zetser et al. showed that the levels of p38 phosphorylation were increased upon HPSE overexpression in MDA-MB-453 and HEK293 cells. In this study, we also showed that overexpression of HPSE lead to increased p38 phosphorylation and HPSE knockdown attenuated the p38 phosphorylation in CRC (**Figure 7C**). In addition, MMP1 has been shown to be tightly regulated by p38 MAPK signaling pathway (17, 26). In our study, inhibition of p38 activity by SB203580 markedly inhibited the induction of MMP1 in HPSE-overexpression cells (**Figure 7C**, right panel). Moreover, strong interactions between p38 and MMP1 demonstrated by protein-protein interaction analysis (**Table S6**). Notably, MMP9 has been reported to be regulated by HPSE in myeloma cells (27), but our RNA-seq results did not show difference in MMP9 expression. Taken together, these results suggested that HPSE might regulate MMP1 expression by p38 MAPK signaling pathway.

Additionally, we performed GO analysis, demonstrating that genes associated with "extracellular matrix organization" in the biological process category were enriched (**Figure 6B**). We further analyzed these DEGs by pathway analysis. Notably, these DEGs are highly enriched in pathway involved in ECM remodeling, including "collagen degradation," "activation of matrix metalloproteinases," "extracellular matrix organization"

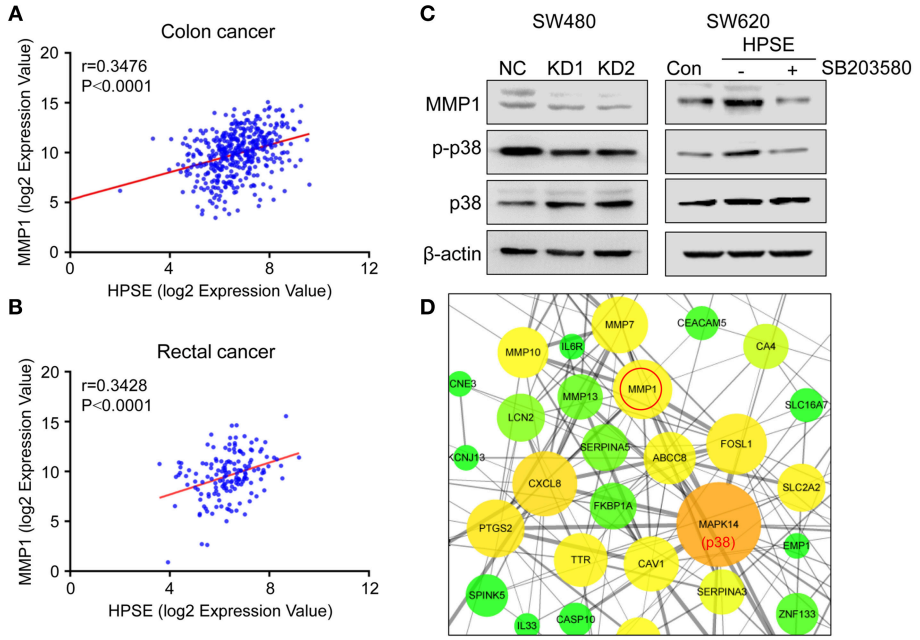

FIGURE 7 | Correlation between the expression of HPSE and MMP1 in colon cancer and rectal cancer. **(A,B)** Positive correlations between the transcriptional levels of HPSE and MMP1 in **(A)** colon cancer and **(B)** rectal cancer. These analyses were performed by analyzing colon and rectal cancer data from TCGA. The correlation between gene expression levels was analyzed by the Pearson correlation test. **(C)** Western blot analysis of MMP1, p38, and p-p38 expression upon knockdown of HPSE (left panel) and overexpression of HPSE (right panel). SW620-HPSE cells were treated with or without p38 pathway inhibitor (SB203580, 10 μM). **(D)** The interactions of the DEGs identified from RNA-seq analysis were extracted from STRING database and visualized using Cytoscape software. The nodes represent proteins and edges represent pairwise interactions. The size of the nodes is proportional to the number of connections established with other genes. The color, from green to red, is used to measure betweenness centrality (BC). The BC quantifies how drastically a gene influences the structure of the whole network. p38 has the largest number of neighboring proteins and MMP1 is tightly linked to p38.

and "degradation of the extracellular matrix," further suggesting the importance of HPSE in CRC invasion and metastasis (**Figure 6A**).

In conclusion, our data indicate that knockdown of HPSE can efficiently inhibit the proliferation, invasion and liver metastasis of CRC cells. Furthermore, RNA-seq analysis revealed that HPSE is tightly linked to the pathways involved in ECM remodeling, and therefore contributes to the invasion and metastasis of CRC. These findings demonstrate that HPSE plays a critical role in the regulation of malignant behavior of CRC cells and suggests that HPSE might be an attractive anti-cancer target in CRC.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://www.cbioportal.org.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medical Animal Care and Welfare Committee of Jining Medical University. The protocol was approved by the Medical Animal Care and Welfare Committee of Jining Medical University.

AUTHOR CONTRIBUTIONS

ZZ and J-WS conception, design, writing and review of the manuscript. XL, WL, JL, and M-JZ acquisition of data. ZZ, SZ, and J-WS analysis and interpretation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00216/full#supplementary-material

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REVIEW
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Next-Generation Cancer Immunotherapy Targeting Glypican-3

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Glypican-3 (GPC3), a 65 kD protein consisting of 580 amino acids, is a heparan sulfate proteoglycan bound to the cell membrane by glycosylphosphatidylinositol. This protein is expressed in the liver and the kidney of healthy fetuses but is hardly expressed in adults, except in the placenta. Contrarily, GPC3 is specifically expressed in hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), yolk sac tumor, and some pediatric cancers. Although the precise function of GPC3 remains unclear, it has been strongly suggested that it is related to the malignant transformation of HCC. We identified GPC3 as a promising target for cancer immunotherapy and have been working on the development of cancer immunotherapeutic agents targeting it through clinical trials. In some trials, it was revealed that the GPC3 peptide vaccines we developed using human leukocyte antigen-A24- and A2-restricted GPC3-derived peptides could induce GPC3-specific cytotoxic T cells in most vaccinated patients and thereby improve their prognosis. To further improve the clinical efficacy of cancer immunotherapy targeting GPC3, we are also developing next-generation therapeutic strategies using T cells engineered to express antigen-specific T-cell receptor or chimeric antigen receptor. In addition, we have successfully monitored the levels of serum full-length GPC3 protein, which is somehow secreted in the blood. The utility of GPC3 as a biomarker for predicting tumor recurrence and treatment efficacy is now being considered. In this review article, we summarize the results of clinical trials carried out by our team and describe the novel agent targeting the cancer-specific shared antigen, GPC3.

Keywords: glypican-3 (GPC3), cancer antigen, cancer immunotherapy, cancer vaccine, cytotoxic T cell, TCR-engineered T cell therapy, CAR-T therapy

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INTRODUCTION

Therapeutic approaches that exploit the immune system are a promising alternative strategy to surgery, radiotherapy, and anticancer drug therapy for cancer treatment. Recent studies have shown that immune checkpoint inhibiters (ICIs) such as antibodies against CTLA-4, programmed cell death (PD)-1, and programmed death ligand 1 have potent and long-term antitumor effects (1, 2); in 2018, Tasuku Honjo and James P Alison won the Nobel Prize for Medicine for their contribution to their development. The extremely high response rates to chimeric antigen

receptor-introduced T cell therapy (CAR-T therapy) for cluster of differentiation (CD)19^{+} hematopoietic tumors and tumor-infiltrative T cell transfer therapy for malignant melanoma have provided further evidence for the efficacy of cancer immunotherapies (3, 4). Additionally, tumor-specific mutant antigens (neoantigen) have attracted attention for their therapeutic potential and clinical trials of personalized cancer vaccines that target neoantigens have been initiated in Europe and the United States (5–7) Meanwhile, peptide vaccines against shared antigens have been developed in Japan but have not yet been approved by the Pharmaceutical Affairs Law.

With the exception of Hodgkin's lymphoma, response rates to ICIs are estimated as no more than 30% in the case of melanoma and 10–20% for other cancers (8, 9). An outstanding challenge is to develop effective therapies for patients who are unresponsive to ICIs. To address this issue, two points must be considered: firstly, the extents to which cancer-responsive T cells are active in patients' bodies; and secondly, how their infiltration into tumors can be enhanced. In cases where cancer-specific effector T cell counts are low, their numbers must be increased by T cell transfer therapy or else the T cells must be induced with cancer vaccines. Inducing inflammation at tumor loci by administration of adjuvants or by chemo- or radiation therapy has been shown to promote T cell infiltration into tumors (10), and ICIs and individualized cancer vaccines derived from neoantigens are thought to be effective in patients with a high frequency of somatic mutations (11). On the other hand, it is difficult to enhance anti-cancer immune responses and even if immunosuppression is overcome (12) in patients with a low, making it necessary to target not only neoantigens but also shared antigens like glypican (GPC)-3. Our clinical trials have shown that cancer peptide vaccines targeting shared antigens can induce peptide-specific cytotoxic T lymphocytes (CTLs) in vaccinated patients without eliciting non-specific autoimmune responses. In addition, gene-based therapy using T cell receptor (TCR) obtained from CTL clones induced by cancer peptide vaccine and isolated from vaccinated patients is expected to have more potent antitumor effects (13). In this review, we summarize the results of cancer immunotherapy targeting GPC3 based on our experience and outline the future prospects.

GPC3

We identified GPC3 in a cDNA microarray screen of several tens of thousands of genes for novel cancer antigens (14). GPC3, 65-kDa protein consisting of 580 amino acids is a heparan sulfate chain proteoglycan bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (**Figure 1A**) (15). GPC3 regulates cell proliferation signals by binding growth factors such as Wnt, fibroblast growth factor, and insulin-like growth factor and plays an important role in the proliferation and differentiation of embryonic cells (16–18). In addition, the gene is present on the X chromosome (Xq26) and shows high homology between humans and mice. Gene mutations and deletions cause gigantism with various malformations and Simpson-Golabi-Behmel syndrome in humans, with similar phenotypic manifestations in mice (16, 19). GPC3 is expressed in various fetal tissues (liver, lung, kidney, and placenta) but is not detected in normal postnatal tissue due to DNA methylation-induced epigenetic silencing (20, 21). On the other hand, GPC3 is expressed in hepatocellular carcinoma (HCC), melanomas, ovarian clear cell carcinoma (OCCC), lung squamous cell carcinomas, and some childhood cancers (hepatoblastomas, nephroblastomas, and yolk sac tumors) (14, 21). Particularly, GPC3 is detected in $\geq$80% of patients with HCC caused by hepatitis B or C (**Figure 1B**) (14, 22, 23). The function of membrane-anchored GPC3 in these cancers is unknown, but it is likely involved in the neoplastic transformation of HCC (23). Additionally, immunohistochemical analysis of HCC tissues have revealed at least three GPC3 expression patterns, which we have classified as diffuse, membrane-localized, and granular (**Figure 1C**) (Shimizu, manuscript in preparation).

Membrane-bound GPC3 can be cleaved and secreted into the blood (**Figure 1A**). Mammalian GPC family members are cleaved at the GPI anchor level by endogenous GPI phospholipase D (24). It was previously proposed that Notum (a conserved secretory feedback inhibitory protein of the Wnt signaling pathway) targets the GPI anchor in a manner similar to phospholipase and draws the GPC/Wnt complex away from the cell surface to inhibit Wnt signaling; however, it is now thought to control the signal without acting on the anchor (25, 26). It is presumed that GPC3 is cleaved between Arg358 and Ser359, which releases the N-terminal region as a soluble protein from cancer cells into the circulation (27). Thus, various forms of GPC3 protein are present in blood, although their functions remain unclear.

Given these features, GPC3 is an ideal target for cancer immunotherapy. We identified each peptide that can bind to human leukocyte antigen (HLA)-A24 or -A2 and induce GPC3 peptide-specific CTLs (28, 29). Furthermore, we conducted clinical trials of vaccines based on these peptides (30–34). In the future, we envision an array of GPC3-based strategies, not only as cancer vaccines, but also in antibody therapy, adoptive immunotherapy with TCR- or CAR-transduced T cells, and others. We also anticipate that plasma GPC3 will be validated as a biomarker for HCC or for evaluating the efficacy of cancer immunotherapy against GPC3.

Preclinical Studies of GPC3-Derived Peptide Vaccine

We have identified HLA-A24- and HLA-A2-restricted GPC3-derived peptides; approximately 60% of Japanese are positive for HLA-A24 and 40% for HLA-A2, which is the major type in Europeans and North Americans (28, 29). Peptide-specific CTLs were induced in mice immunized with these GPC3 peptides, which exerted antitumor effects without eliciting an autoimmune response (28, 29). In preparation for clinical trials using these HLA-restricted peptides, we investigated whether differences in peptide dosage would affect the efficacy of vaccination, and performed studies in mice to determine the optimal adjuvant (35). We compared five groups: peptide-only and peptide combined with incomplete Freund's adjuvant (IFA), CpG, α-GalCel, or aluminum and found that the GPC3-specific CTLs were only induced in the IFA combination group, demonstrating that the peptide alone was ineffective. We therefore used the

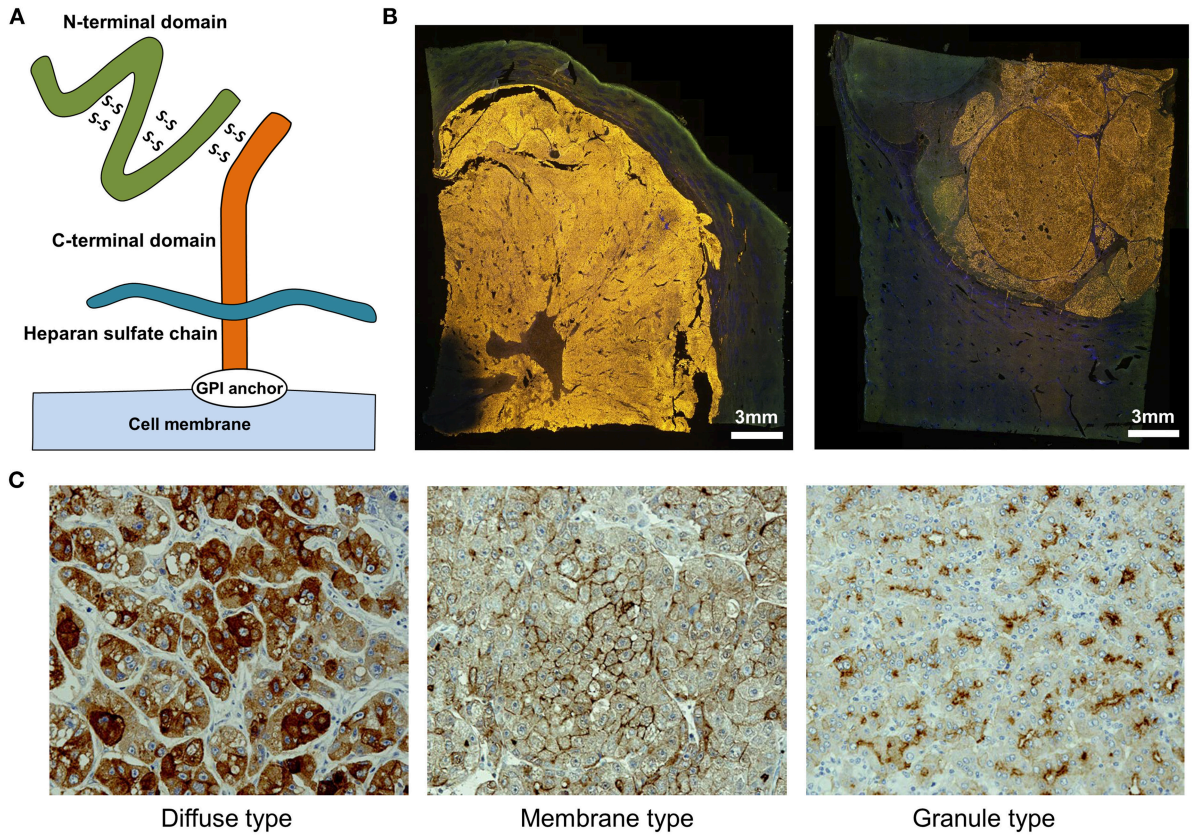

FIGURE 1 | Characteristics of GPC3. **(A)** Schema of GPC3. **(B)** Fluorescence micrographs of GPC3-positive HCC. GPC3 labeling appears as a yellow color. **(C)** GPC3 expression patterns can be classified into diffuse, membrane, and granule types by immunohistochemistry.

peptides plus IFA as cancer vaccines in clinical trials and observed that stronger immune responses were induced by varying peptide dosage.

Phase I Clinical Trial of GPC3 Peptide Vaccines Against Advanced HCC

At the National Cancer Center Hospital East (Kashiwa, Japan), we conducted a phase I clinical trial of GPC3 peptide vaccines in a cohort comprising 33 cases of advanced HCC from February 2007 to November 2009 (UMIN Clinical Trials Registry: 000001395) (**Table 1**) (30, 38). The primary endpoints were safety and immune response. In a dose escalation study of 0.3, 1, 3, 10, and 30 mg there was no dose-limiting toxicity (DLT), making it difficult to determine the maximum tolerated dose. Although the partial clinical response in a patient treated with 30 mg as well as dose-dependent immunological reactions suggested a greater efficacy of high dosages, a dose of 30 mg required a total vaccine volume of 6 ml, which was difficult to administer and caused pain along with reddening and induration at the site of administration. Based on these observations, we determined that a dosage of 3 mg would be appropriate for the next-phase trial.

In this first-in-human study of GPC3 peptide vaccines, we confirmed the safety of GPC3 peptide vaccines and obtained promising clinical results. We also detected an elevation in peptide-specific CTL counts in peripheral blood by interferon-γ enzyme-linked immunosorbent spot assay, demonstrating that these vaccines can induce immune responses even in humans. By analyzing tumor biopsies we identified cases where infiltrating $CD8^+$ CTLs were present after but not before vaccination, confirming the immunological effects of our vaccines (30, 38).

A subsequent phase I trial was initiated to investigate the extent of CTL infiltration into tumor tissues (UMIN Clinical Trials Registry: 000005093) by analyzing tumor biopsies obtained before and after vaccination (**Table 1**) (33). The primary endpoint was GPC3 peptide-specific immune response induced by vaccination. However, this trial was conducted after approval of sorafenib treatment, and patients with extremely late-stage HCC were registered only after sorafenib had ceased to be effective; indeed, most of the patients showed negligible response to the vaccine, probably because of their endogenous immunosuppressive states. Additionally, post-vaccination biopsies were completed in just 11 cases. Nevertheless, we were able to glean useful data from this trial: in one case the HCC tissue became inflamed and then necrotic after two injections of the vaccine, despite ongoing liver dysfunction (39); and we established GPC3 peptide-specific CTL clones from a tumor biopsy specimen (33).

TABLE 1 | Summary of clinical trials for cancer immunotherapy targeting GPC3.

Trial	ID	References	Key inclusion criteria	Primary endpoint	Results
OUR CLINICAL TRIALS					
Phase I clinical study of GPC3 peptide vaccine in patients with advanced HCC	UMIN 000001395	Sawada et al. (30)	Advanced HCC patient	(1) Adverse effects of GPC3 vaccine	GPC3 vaccination was well-tolerated; the vaccine induced a GPC3-specific CTL response in 30/33 patients (91%)
				(2) GPC3-specific immune responses to GPC3 vaccine	
Clinical study evaluating immunological efficacy of GPC3 peptide vaccine in patients with advanced HCC	UMIN 000005093	Tsuchiya et al. (34)	Advanced HCC patient	Increased percentage of GPC3 peptide-specific CD8-positive T lymphocytes in blood and tumor	After vaccination, the number of GPC3 peptide-specific CTLs in PBMC was increased in 9 of 11 patients; tumor biopsy specimens obtained from three patients post-vaccination revealed CTL infiltration
Phase II study of GPC3 peptide vaccine as adjuvant treatment for HCC after surgical resection or RFA	UMIN 000002614	Sawada et al. (31)	(1) Diagnosed as initial HCC	1- and 2-year recurrence rate	1- and 2-year recurrence rates were 24.4 and 53.7%, respectively; the primary endpoint was not reached
			(2) Subjects who underwent potentially curative surgical resection or RFA for treatment of HCC		
Phase II study of GPC3 peptide vaccine for treatment of OCCC	UMIN 000003696	Suzuki et al. (32)	Advanced OCCC patient	DCR at 6 months	DCR at 6 months was 9.4% (3/32)
Phase I study of GPC3 peptide vaccine for pediatric patients with refractory tumors	UMIN 000006357	Tsuchiya et al. (33)	(1) Patients with refractory, recurrent, or progressive status (progressive group)	Incidence of DLT	No DLT or dose-specific adverse events were observed
			(2) Patients in remission without chance of cure (remission group)		
			(3) Patients in partial remission or with stable disease (partial remission group)		
OTHER CLINICAL TRIALS					
First-in-man phase I study of GC33, a novel recombinant humanized antibody against glypican-3, in patients with advanced hepatocellular carcinoma	NCT 00746317	Zhu et al. (36)	Patients with measurable, histologically demonstrated advanced HCC	Maximum tolerated dose was not reached as there was no DLT up to the highest planned dose level. Median TTP was 26.0 and 7.1 weeks in the high and low GPC3 expression groups, respectively	
Japanese phase I study of GC33, a humanized antibody against glypican-3 for advanced hepatocellular carcinoma	JapicCTI 101255	Ikeda et al. (37)	Japanese patients with advanced HCC	No DLT observed in any patient up to the highest planned dose; 7/13 patients showed SD, 6/13 showed PD, and 3/13 showed long-term SD >5 months	

CTL, cytotoxic T lymphocyte; DCR, disease control rate; DLT, dose-limiting toxicity; GPC3, glypican-3; HCC, hepatocellular carcinoma; OCCC, ovarian clear cell carcinoma; PBMC, peripheral blood mononuclear cell; PD, progressive disease; RFA, radiofrequency ablation; SD, stable disease; TTP, time to progression.

Phase II Clinical Trial to Investigate Relapse Prevention Following Radical Treatment of HCC

We performed a single-arm phase II clinical trial to evaluate 1- and 2-year relapse rates in 41 cases following radical treatment of HCC using GPC3 peptide vaccine as adjuvant therapy (**Table 1**) (31). GPC3 peptide-specific CTL responses were detected in 35 of the 41 patients (85.4%) after vaccination. Since the absence of GPC3 expression is correlated with good prognosis, we compared patients with GPC3-positive HCC and control subjects and found that post-surgical administration of GPC3 peptide vaccine can extend the recurrence-free survival period; moreover, overall survival was prolonged in cases where CTL

induction was observed. This is likely due to the suppression of GPC3-positive HCC with highly malignant features. We plan to report these results in the future (Miura, manuscript in preparation). Meanwhile, there were two cases of relapse despite the presence of numerous induced peptide-specific CTLs in peripheral blood due to vaccine administration. In these cases, GPC3 was expressed in the primary tumor, but in recurrent cancer, the expression was undetectable (31). These observations suggest that peptide vaccine targeting one type of shared antigen—which could eliminate tumor cells expressing the antigen—may not completely prevent tumor growth due to the increased heterogeneity of the cancer. In such instances cancer peptide vaccines that target multiple shared antigens or neoantigens may be more effective. Furthermore, in nine patients whose recurrent tumors expressed GPC3, the frequencies of GPC3-specific CTLs tended to be lower than those in the aforementioned two patients. Although peptide-specific CTLs were induced by vaccination, the reduction of GPC3-positive HCC recurrence due to the peptide vaccine might depend on the strength of CTL induction. We also considered identifying helper T (Th) cell epitopes in the vaccinated patients (40). Our study revealed that GPC3-derived long peptides-specific and HLA class II-restricted CD4± T-cell responses were observed in 14 of 20 patients, and the presence of the specific Th cells was correlated with prolonged overall survival (40). We expect clinical trials to confirm the recurrence prevention effects of peptide vaccines after resection in patients with GPC3-positive HCC.

Clinical Trials for Advanced OCCC

We also observed the antitumor effects of GPC3 peptide vaccine in advanced OCCC in clinical trials performed at Nagoya University (UMIN Clinical Trials Registry: 000003696) (**Table 1**) (32, 41). The primary endpoint was disease control rate (DCR) at 6 months. Two cases showed partial response (PR) and one showed stable disease (SD); DCR at 6 months was 9.4% (3/32 cases). While response rates tended to be higher than for HCC, this may be due to differences in tumor quantity. OCCC is extremely difficult to cure with existing anticancer drugs, lending urgency to the development of effective cancer immunotherapies.

Clinical Trials for Refractory Pediatric Cancer

As mentioned above, GPC3 is expressed in some pediatric cancers, including hepatoblastoma, nephroblastoma (Wilms' tumor), and yolk sac tumors. We performed a multicenter clinical trial that included GPC3-positive refractory pediatric cancer cases (UMIN Clinical Trials Registry: 000006357) (**Table 1**) (34); 18 patients received GPC3 peptide vaccination. DLT—the primary endpoint—was not observed, and the vaccine induced a GPC3-specific CTL response in 7/18 patients (39%), nearly all of whom belonged to the remission group and were hepatoblastoma patients. In contrast, GPC3-specific CTL frequency was not increased in the refractory advanced progression group. These results suggest that the vaccine can prevent recurrence of hepatoblastoma after the second remission, a period in which relapse is generally considered as unavoidable. Furthermore, recurrence-free survival of more than 4 years was observed in all five patients with hepatoblastoma.

TABLE 2 | Summary of clinical trials for cancer immunotherapy targeting GPC3.

Trial	Patients	ID
A randomized, placebo-controlled, double-blind, multicenter phase II trial of intravenous GC33 at 1,600 mg Q2W in previously treated patients with unresectable advanced or metastatic HCC	Histologically confirmed HCC	NCT 01507168
A phase I dose escalation and cohort expansion study of ERY974, an anti-GPC3/CD3 bispecific antibody, in patients with advanced solid tumors	Patients with GPC3-positive advanced solid tumors not amenable to standard therapy or for which standard therapy was not available or not indicated	NCT 02748837
A phase I study of anti-GPC3 chimeric antigen receptor modified T cells in Chinese patients with refractory or relapsed GPC3 + HCC	Patients with GPC3-positive HCC	NCT 02395250
Glypican 3-specific chimeric antigen receptor expressing T cells as immunotherapy for patients with HCC	Patients with GPC3-positive HCC	NCT 02905188

GPC3, glypican-3; HCC, hepatocellular carcinoma.

Intratumoral Vaccination Therapy

Tumor cells reduce their antigen presentation to escape host immune surveillance mechanisms (42), which is a major challenge in the development of effective cancer vaccines. HLA class I expression is reduced or absent in 16–50% of various malignancies (43). To circumvent this problem, we developed an intratumoral injection method for peptide vaccines that has been tested in mice. This mode of delivery enhanced anti-tumor activity as compared to conventional subcutaneous injection and induced systemic immune responses that inhibited the growth of metastasized tumors (44). Moreover, combining intratumoral peptide vaccine injection and anti-PD-1 blocking antibody could elicit enhanced antitumor effects by inducing the upregulation of PD-1 on the surface of CTLs (45–47). This may be applicable not only to primary tumors but also to distant metastatic sites, which could be targeted by loading peptide into HLA class I of tumor cells.

Therapy With GPC3 Peptide-Specific CTL Clones Established From Vaccinated Patients

In our clinical trials of GPC3 peptide vaccine, we successfully established multiple types of GPC3 peptide-specific CTL clones derived from the peripheral blood and cancer tissue of vaccinated patients (34, 38, 48). Some of these clones have a strong ability to kill cancer cells presenting GPC3 peptide *in vitro*. By cloning these TCRs, we are currently developing an adoptive immunotherapy approach based on these TCR-transduced T cells. GPC3 peptide-specific TCRs were cloned from CTLs obtained from patients who showed no adverse reactions to

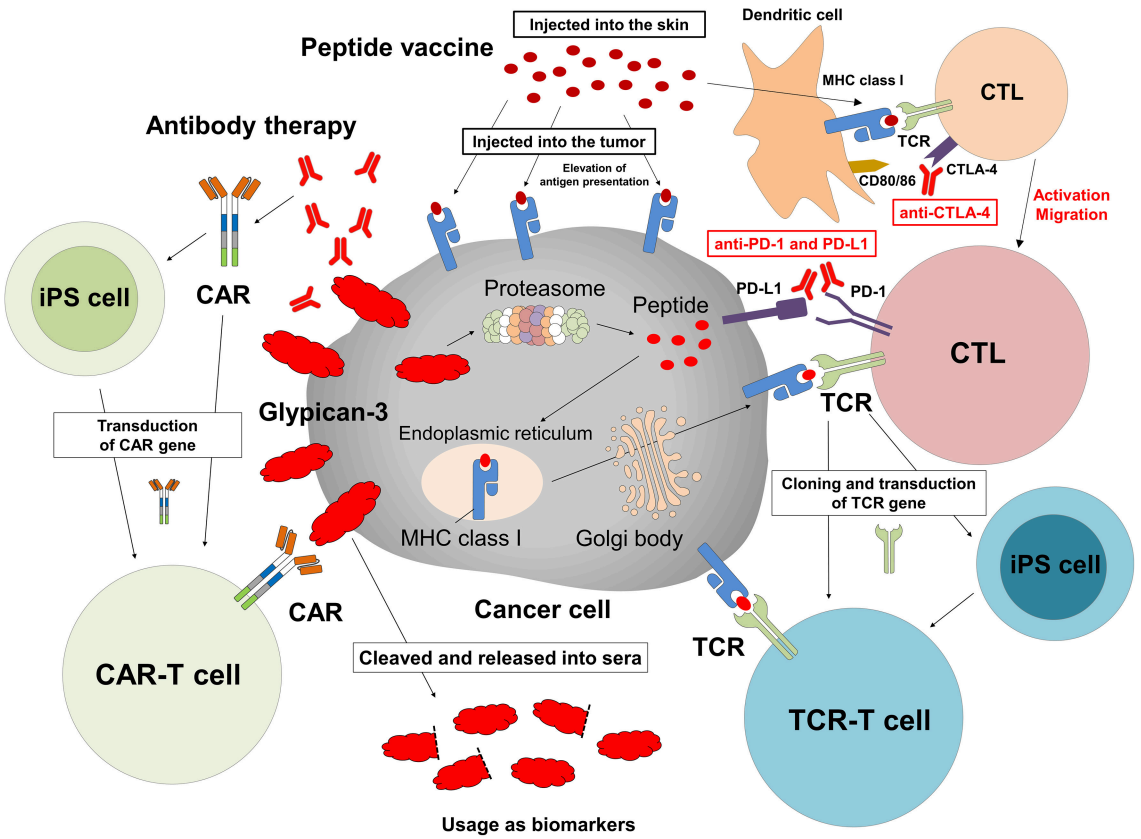

FIGURE 2 | Cancer immunotherapy targeting GPC3. Therapeutic approaches that target intracellular GPC3 include GPC3 peptide vaccine and adoptive immunotherapy based on T cells transduced with a suitable TCR. In contrast, antibody therapy and anti-GPC3-CAR-transduced T cell therapy target membrane-bound GPC3. We are currently developing such T cells and CAR-T cells from iPS cells. In addition, intratumoral vaccination and combination with immune checkpoint inhibitors might enhance effects of these treatments, and serum GPC3 could be a biomarker.

the peptide vaccine other than reddening and local swelling at the administration site, thus guaranteeing vaccine safety. Adoptive immunotherapy with TCR-transduced T cells is generally considered as having superior antitumor effects to peptide vaccine therapy, and their application to advanced cancers presenting GPC3 peptide is highly anticipated.

Antibody Therapy Targeting Membrane GPC3

GC33, a humanized monoclonal antibody against GPC3, has been shown to induce antibody-dependent cell-mediated cytotoxicity against GPC3-positive HCC cell lines and elicit anti-tumor effects in patient-derived xenograft cancer models (49). In a first-in-human phase I trial performed in the United States for patients with advanced HCC, GC33 was well-tolerated and antitumor effects were observed in some patients with high GPC3 expression HCC (**Table 1**) (36). A phase I trial of GC33 carried out in Japan confirmed its tolerability. While there were no complete response or PR cases, SD was achieved by 7 of 13 patients who received the treatment, three of whom maintained this status for 3 or more months (**Table 1**) (37). A randomized, placebo-controlled, double-blind, multicenter phase II trial of GC33 is presently underway (**Table 2**). In addition, the results of a basic research study on ERY974, an anti- GPC3/CD3 bi-specific T cell-redirecting antibody were recently reported (50). A phase I clinical trial to confirm its toxicity is currently underway in which there is no limitation to the cancer type if the primary tumor is GPC3-positive (**Table 2**).

Development of CAR-T Therapy Targeting Membrane GPC3

While CAR-T therapy has shown a remarkable response rate exceeding 80% against B cell blood tumors (51, 52), fully promising results have not yet been obtained against solid carcinomas, such as glioblastoma (53), pancreatic cancer (54), and prostate cancer (55, 56). These insufficient responses were thought to be caused by tumor heterogeneity, immunosuppressive mechanism in the tumor microenvironment, and insufficient accumulation of CAR-T cells in the tumor (57). One option for ameliorating these problems is IL-7/CCL19 expressing CAR-T cells (58). These cells promoted the migration and activation of not only CAR-T cells but also T cells and dendritic cells at the tumor locus, thereby demonstrating strong anti-tumor effect on solid tumors in mice

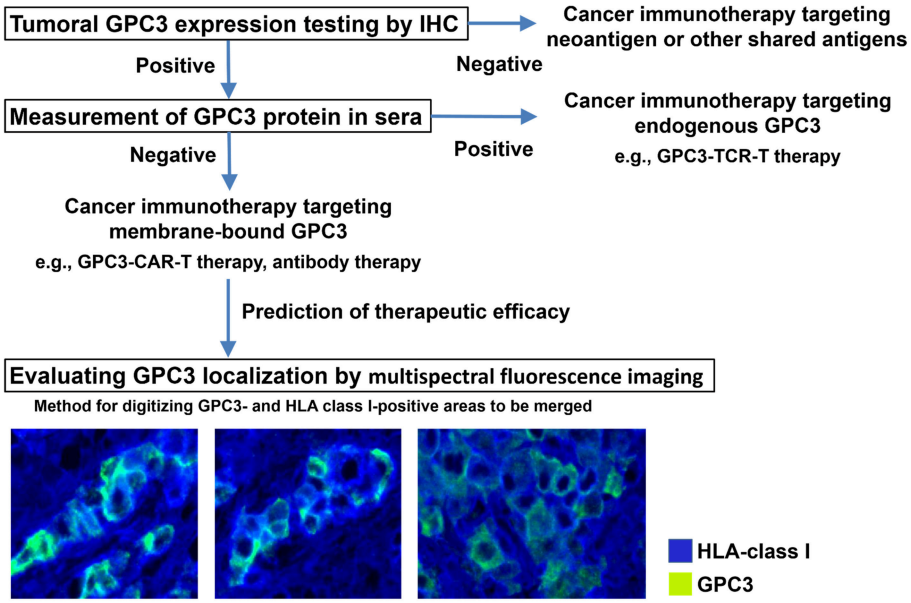

FIGURE 3 | Appropriate treatments targeting GPC3. Analyzing tumor GPC3 expression in combination with serum GPC3 level can reveal the most effective therapeutic strategy. To determine whether GPC3 is predominantly expressed at the cell membrane—which is thought to influence the efficacy of therapies targeting membrane-bound GPC3—we are developing a method of digitizing GPC3- and HLA class I-positive areas to be merged using a multispectral fluorescence imaging system.

(58). Moreover, in order to avoid on-target off-tumor toxicity, it is necessary to select an excellent cancer antigen with high tumor specificity. In this regard, GPC3 is considered an ideal target as described above. The development of CAR-T therapy targeting solid tumors is underway around the world, and therapies based on GPC3 antibody gene (GPC3-CAR) have been developed (59, 60). In China and United States, clinical trials of GPC3-CAR therapy against GPC3-positive HCC have already begun (**Table 2**). By combining it with new technologies that supplement the weakness of CAR-T therapy, it is desired that the clinical effect against solid tumors would be further improved.

Development of a Novel Immunotherapy Using Induced Pluripotent Stem (iPS) Cells

We think that there is significant value to using T cells derived from iPS cells for the following three reasons: (i) they eliminate the effects of effector T cell exhaustion and aging; (ii) they enable reliable gene manipulation at the iPS cell stage; and (iii) they make it possible to continue the treatment over a long period of time. By using an iPS cell bank, we may be able to develop innovative adoptive immunotherapeutics that can be universally adopted at a low cost. In addition, since some patients may have T cells that lack intrinsic anti-tumor potency, an alternative, third party off the shelf T cell product might be a good alternative. Kaneko et al. at Kyoto University in Japan have successfully prevented unwanted reconstruction of TCRs by excluding genes causing TCR remodeling in T cells derived from iPS cells through genome editing, and showed that cytotoxic T cells with high avidity for cancer cells can be induced through this process (61). We are currently developing an iPS cell-derived TCR-T cell (TCR-iPS-T cell) strategy for expressing GPC3-peptide specific TCR in collaboration with their team. In detail, the cell was created by transducing HLA haplotype homologous iPS cell clones with HLA-A*24:02 restricted $GPC3_{298-306}$ peptide-specific TCR gene using a lentiviral vector and then stimulating differentiation (61). The findings to date indicate that these TCR-iPS-T cells derived from an HLA homozygous iPS cell stock showed both cancer antigen-specific cytotoxic effects caused by CAR and non-specific cytotoxicity due to stimulation of natural killer cell ligands against GPC3-positive cell lines. These results may lead to the development of a novel type of immunotherapy that can prevent the suppression of anti-cancer effects caused by immune editing against a particular antigen.

Biomarkers Based on Serum Full-Length GPC3

As described above, GPC3 is released into the serum of HCC patients, and its utility as a tumor marker has been reported (62, 63). We established a novel sandwich enzyme-linked immunosorbent assay system for predicting HCC recurrence after surgery based on post-operative elevation of serum GPC3 level (64). In immunohistochemical analyses of GPC3-positive HCC specimens, a few surrounding normal cells also weakly expressed GPC3, which we believe contribute to post-operative GPC3 secretion and recurrence (64). In partnership with a private company, we have developed an assay to quantify serum full-length GPC3 level, which is the most physiologically relevant parameter. In combination with existing tumor markers such as alpha-fetoprotein and protein induced by vitamin K absence II,

we successfully predicted early recurrence of HCC after surgery (Miura, manuscript in preparation). We are presently examining whether this assay can predict hepatocellular carcinogenesis in patients with chronic hepatitis and cirrhosis as well as response to anti-GPC3 therapy.

Companion Diagnosis for Cancer Immunotherapy Against GPC3

We are in the process of developing various treatments and diagnostic methods targeting GPC3 (**Figure 2**) (65). The subcellular localization of GPC3 and its presence of serum are expected to affect the effects of each treatment approach. Soluble full-length GPC3 protein could block anti-GPC3 antibody and CAR-T cells, thereby reducing cytotoxicity and leading to unexpected side effects. Based on these considerations, serum GPC3 could serve as a biomarker for evaluating treatment effect or be used to assess the eligibility of patients for antibody or GPC3-CAR-T therapy. In addition, the clinical trial for GC33 revealed a correlation between the localization of GPC3 and clinical effects (36). We have also described multiple distinct GPC3 expression patterns (**Figure 1C**). Therefore, it is important to analyze the localization of GPC3 and not only its presence or absence. To this end we are currently developing a method for investigating the co-expression of GPC3 and class I HLA—a cell membrane-associated molecule—by multiple immunolabeling that can be used as a companion diagnostic tool (**Figure 3**).

FUTURE PERSPECTIVES

GPC3 has unprecedented cancer specificity and is being studied as a target for cancer immunotherapy worldwide. However, there remain many open questions regarding its natural history, function, and dynamics. Clarifying these aspects of GPC3 is necessary for the development of more effective treatments. Since Boon et al. first identified a cancer-specific antigen for melanoma in 1991, many studies have been undertaken to search for new cancer antigens (66). We identified GPC3 as a tumor-associated antigen and have developed GPC3 peptide vaccine as cancer immunotherapy. Based on findings from our clinical trials, we are now developing the next generation of GPC3-targeting therapeutic approaches such as CAR-T and TCR-engineered T cell therapy. On the other hand, neoantigen is gaining global attention, with significant advances in the establishment of immunotherapies targeting these molecules. We are also developing personalized cancer immunotherapies such as cancer vaccines using peptides derived from neoantigens or individualized adoptive T cell therapies using next-generation sequencers. We look forward to advances in research on neoantigens or shared antigens such as GPC3 that can demonstrate which of these can best serve as immunotherapeutic targets.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Leukocyte Heparanase: A Double-Edged Sword in Tumor Progression

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Heparanase is a β-D-endoglucuronidase that cleaves heparan sulfate, a complex glycosaminoglycan found ubiquitously throughout mammalian cells and tissues. Heparanase has been strongly associated with important pathological processes including inflammatory disease and tumor metastasis, through its ability to promote various cellular functions such as cell migration, invasion, adhesion, and cytokine release. A number of cell types express heparanase including leukocytes, cells of the vasculature as well as tumor cells. However, the relative contribution of heparanase from these different cell sources to these processes is poorly defined. It is now well-established that the immune system plays a critical role in shaping tumor progression. Intriguingly, leukocyte-derived heparanase has been shown to either assist or impede tumor progression, depending on the setting. This review covers our current knowledge of heparanase in immune regulation of tumor progression, as well as the potential applications and implications of exploiting or inhibiting heparanase in cancer therapy.

Keywords: heparanase, leukocytes, macrophages, natural killer cells, immunotherapy, tumor progression

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INTRODUCTION

Heparanase is the only mammalian enzyme that directly cleaves heparan sulfate side chains of heparan sulfate proteoglycans (HSPGs), key components of the extracellular matrix and basement membrane. The cleavage of heparan sulfate by heparanase regulates a number of fundamental cellular processes including cell migration (1, 2), cytokine production (3, 4), angiogenesis (5), and wound healing (6). Furthermore, heparanase has also been implicated in cell adhesion that is independent of its enzymatic activity (7, 8). The ability of heparanase to regulate these processes also makes it a key player in several pathological settings such as inflammatory disease and cancer. Heparanase contributes to various inflammatory diseases including delayed hypersensitivity, vascular injury, chronic colitis, Crohn's disease, sepsis, rheumatoid arthritis (9), atherosclerosis (10), and diabetes (11–13). Furthermore, heparanase is upregulated in response to pro-inflammatory cytokines, bacterial or viral infections, and modulates innate immune cell function. For example, in sepsis heparanase is upregulated by tumor necrosis factor-α (TNF-α) and induces shedding of the glycocalyx, thereby exposing the endothelial surface and adhesion molecules which facilitate neutrophil recruitment (14). Heparanase has also been well-characterized in cancer (15, 16), where the overexpression of heparanase often contributes to tumor progression (17, 18). The overexpression of heparanase has been detected in almost all cancer types, where it promotes metastasis (19–21), angiogenesis (19, 21, 22), and tumor proliferation (23). More

recently, the role of leukocyte heparanase in tumor progression has been more closely examined, with the suggestion that it can be either pro- or anti-tumorigenic, depending on the setting.

HEPARANASE EXPRESSION BY LEUKOCYTES

The first documentation of heparanase expression in leukocytes was in T lymphocytes where the production of an endoglycosidase was observed in assisting their migration and penetration of the basement membrane and blood vessel entry (24). Subsequently, heparanase expression has been further characterized in T cells (25–30) as well as a number of other leukocytes including B cells (31), natural killer (NK) cells (2), monocytes (32), dendritic cells (DCs) (1, 32), macrophages (29, 30, 33–37), neutrophils (38–40), mast cells (41), and eosinophils (42). The expression of heparanase by leukocytes is inducible by various cell activatory stimuli (2, 43, 44) and has been shown to promote leukocyte migration (1, 45), cell rolling and adhesion (46, 47), the upregulation of pro-inflammatory cytokines (3), and activation of innate immune cells (34). Heparanase has also been associated with inflammatory diseases such as atherosclerosis (10) and diabetes (11–13). However, despite this progress, much remains to fully understand the role of heparanase in leukocytes and its contribution to disease. It is well-established that leukocytes are important regulators of tumor progression (48–51). An emerging area of significant clinical interest is at the intersection of heparanase, leukocytes, and cancer. We will now discuss how heparanase may regulate leukocyte function in the context of tumor progression and its relevance in cancer therapy.

LEUKOCYTE HEPARANASE AND TUMOR PROGRESSION

Heparanase and Macrophage Activation and Infiltration Into Tumors

Tumor associated macrophages (TAMs) are often found within primary tumors and pre-metastatic sites, and their presence frequently contributes to tumor progression (52, 53). In heparanase knockout mice, macrophage infiltration into implanted Lewis lung carcinoma tumors was impaired, and tumors were smaller than in wild type animals (34). Macrophages from heparanase knockout mice also expressed lower levels of the pro-inflammatory cytokines TNF-α, interleukin-1 β (IL-1β), C-X-C motif chemokine ligand 2 (CXCL2) and IL-6 (34, 54). The opposite was observed in a model of pancreatic cancer overexpressing heparanase. Pancreatic tumor cells overexpressing heparanase were implanted into severe combined immune deficiency (SCID) mice, which lack B and T cells (55). Implanted tumors with heparanase-overexpressing pancreatic cancer cells were observed to have more infiltrating macrophages and larger tumors compared to tumors with normal heparanase expression (54) (**Figure 1A**). The overexpression of heparanase in these pancreatic tumors also led to increased macrophage expression of IL-6, IL-10, C-C motif chemokine ligand-2 (CCL-2), vascular endothelial growth factor (VEGF) and macrophage scavenger receptor-2 (MSR-2) (54) (**Figure 1A**). Indeed, TAM expression of these cytokines is an indicator of macrophage polarization to an M2 phenotype, which facilitates tumorigenesis (52, 56, 57). These findings suggest that both tumor-derived and macrophage-derived heparanase can promote the recruitment of macrophages to tumors and facilitate their entry to aid tumor progression.

During inflammation and inflammation-associated tumorigenesis, the source of heparanase is often the epithelium (58). This was identified in patients with inflammatory bowel disease (IBD) (59), and in an IBD model, epithelial cell-heparanase was found to drive the over-activation of macrophages, inflammation, and ultimately tumorigenesis (60). In this model of IBD, heparanase-overexpressing mice were also observed to have more macrophages in the colon when compared to wild type animals (60). This overexpression of heparanase in the epithelium has been characterized in other models of inflammation, including pancreatitis (61) and Barrett's epithelium in the esophagus (62). However, it remains to be explored whether epithelial cell-heparanase in these settings also influences immune cell activation. Furthermore, another study found that recombinant heparanase added to colorectal cancer cell lines could increase mRNA expression and release of monocyte chemoattractant protein-1 (MCP-1) (63), supporting the notion that heparanase may help to generate a chemokine gradient to recruit macrophages to sites of inflammation.

Together, these findings show that heparanase from the tumor cells, macrophages, and epithelial cells can promote tumorigenesis. However, not all tumor cells overexpress heparanase. Weissmann et al. found that Raji lymphoma cells expressed low levels of heparanase *in vitro*, but when implanted into mice, exhibited increased heparanase activity (22) (**Figure 1B**). This may have been a result of the tumor cells upregulating heparanase in response to stimuli from the tumor microenvironment, which could include soluble factors such as TNF-α and IL-1β (35, 43), or heparanase may have originated from other cell types within the tumor microenvironment (e.g., macrophages). Regardless of the source of heparanase, its inhibition with the heparanase neutralizing antibody Ab 1453 in these tumors was sufficient to reduce tumor growth (22). Again, the source of heparanase in this example is unclear, but this study supports the idea that tumor cells can utilize heparanase from the tumor microenvironment with similar outcomes on tumor progression.

Tumor Cells Modulate Heparanase Expression in Lymphocytes

Tumor cells can influence leukocyte function via direct cell-cell interaction (64), or through secreted factors (65). A study by Theodoro et al. found that lymphocytes from peripheral blood mononuclear cells (PBMCs) of breast cancer patients displayed higher heparanase expression than lymphocytes from healthy patients (66). The study also found that heparanase expression was higher in lymphocytes from patients with metastases, and that heparanase expression in circulating lymphocytes was

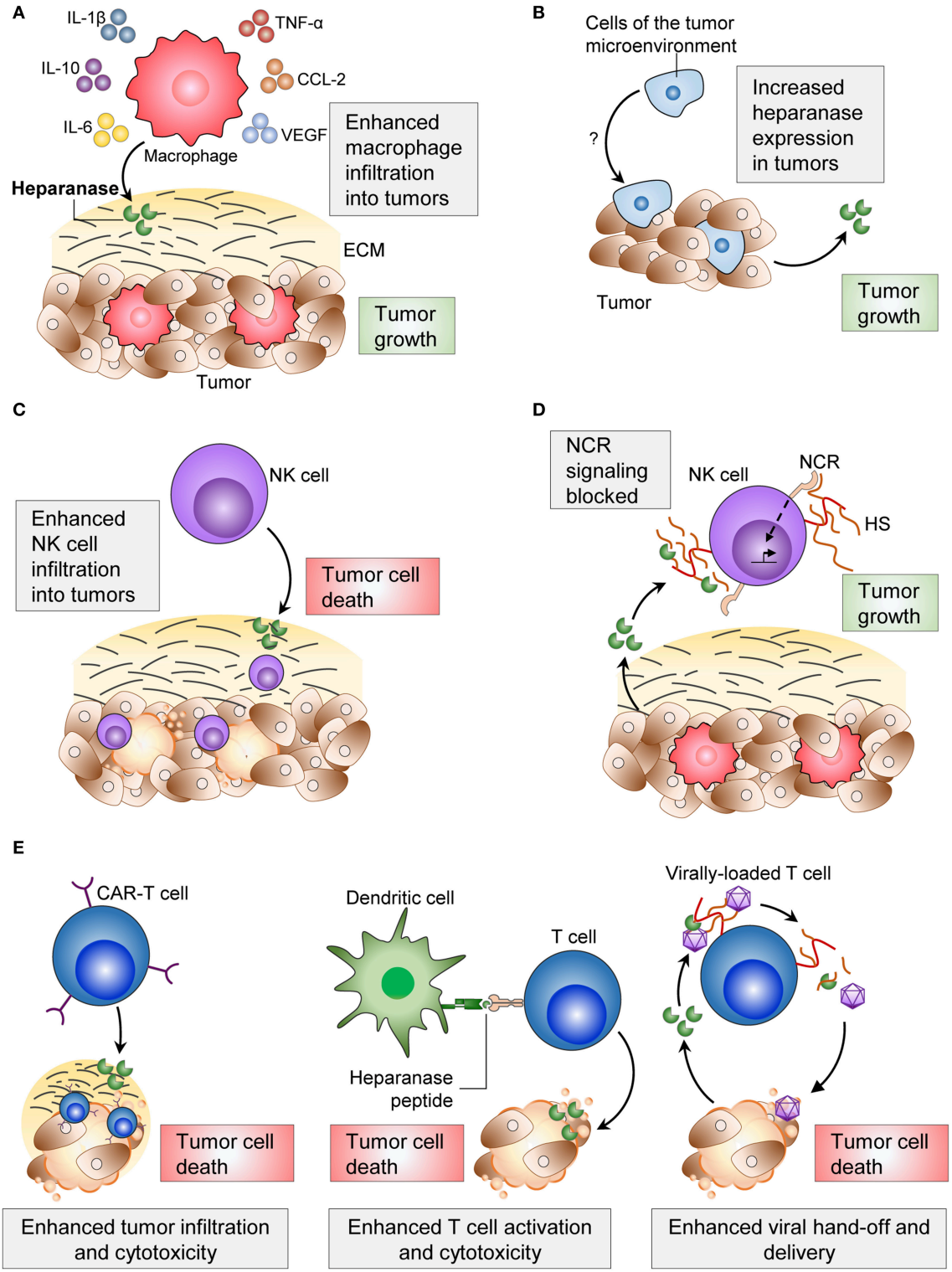

FIGURE 1 | Effects of heparanase on immune cells and the consequences on tumor progression. **(A)** Heparanase from macrophages and from tumor cells increase macrophage infiltration into tumors, cytokine secretion and phagocytic ability. **(B)** Cells of the tumor microenvironment increase tumor cell-heparanase and increase tumor cell proliferation. **(C)** Heparanase enhances NK cell infiltration into tumors and consequent tumor cell clearance. **(D)** Tumor cell-heparanase can block NCR signaling and consequent activation of NK cells. **(E)** Applications of immune cell-heparanase include use in CAR T cells, dendritic cell vaccines, and viral delivery of anti-tumorigenic agents. ECM, extracellular matrix; NCR, natural killer cytotoxicity receptor; HS, heparan sulfate; CAR-T, chimeric antigen receptor-T.

reduced following surgical resection of tumors (66). Breast tumor cells when co-cultured with lymphocytes from healthy donors were shown to induce heparanase expression by the lymphocytes. Furthermore, these experiments suggested that the breast tumor cells induced the lymphocytes to produce soluble factors that were responsible for upregulating heparanase expression (66). It was proposed that by increasing expression of heparanase in tumor-infiltrating lymphocytes, the tumor would have the ability to alter gene expression of many other neoplastic and non-neoplastic cells (66). The impact of these high-heparanase expressing lymphocytes was not tested. However, since these patients had higher instances of metastasis, it suggests that these lymphocytes may be preparing to seek out tumor cells for clearance, given that heparanase is often upregulated upon T cell activation (24, 25, 27, 28).

Heparanase and NK Cell-Mediated Clearance of Tumors

NK cells efficiently kill tumor cells of many origins, and their presence within tumors often correlates with improved survival (67). We recently reported that mice deficient in NK cell-heparanase exhibited reduced NK cell tumor infiltration, resulting in impaired clearance of B16F10 melanoma tumors and metastases (2) (**Figure 1C**). Furthermore, immune checkpoint inhibitors targeting the programmed death ligand-1 (PDL-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) axes were less effective in the absence of NK cell-heparanase (2). These data suggest that using heparanase inhibitors concomitantly with checkpoint inhibitors would be ineffective. To our knowledge, this is the first description of a tumor suppressive role for heparanase.

Heparanase Blocks NK Cell Activation

The role of heparanase in NK cell function does not appear to be simply in cell migration and invasion, but may also regulate NK cell activation and cytotoxicity.

Heparan sulfate on the plasma membrane of NK cells can act in a *-cis* manner as a co-ligand for the NK cell cytotoxicity receptor (NCR) (68). However, surface heparan sulfate must compete with soluble or *-trans* heparan sulfate for NCR binding, which dampens NK cell activation. By cleaving heparan sulfate on the surface of NK cells, heparanase secreted by tumors can increase levels of soluble heparan sulfate, and consequently inhibit NK cell activity and cytotoxicity against tumor cells (69) (**Figure 1D**). It appears that low levels (69) or high levels (68) of *-cis* heparan sulfate-NCR interaction dampens NK cell activation, and that maintaining optimal levels of membrane-bound heparan sulfate is important for optimizing NK cell activation.

Despite these advances, further investigation is required to fully understand the role of heparanase in leukocyte function during cancer progression. Given the ability of heparanase to modulate pro-inflammatory cytokine levels (3, 60), and activate and recruit tumor-promoting leukocytes (34, 54), it is likely that heparanase plays a greater role in modulating the immune system and immune suppression during cancer progression.

EXPLOITING HEPARANASE IN CANCER IMMUNOTHERAPY

Despite the complex pro/anti-tumorigenic axis of heparanase, exploiting heparanase has shown promise in leukocyte-based anti-cancer therapies.

Heparanase in CAR-T Cell Therapy of Solid Tumors

Chimeric antigen receptor (CAR)-T cell therapy utilizes engineered recombinant receptors expressed on T cells containing an antigen-recognition domain of a monoclonal antibody and a T cell-activating domain (70). These CARs enable T cells to specifically and efficiently recognize tumor cells and maximize T cell function. Whilst this therapy has shown promise in many hematological malignancies (71, 72), it is relatively ineffective against solid tumors, partly attributed to the low penetration of CAR-T cells into the tumor (70). To address this, Caruana et al. overexpressed heparanase in human CAR-T cells, and found this to assist CAR-T cell infiltration into neuroblastoma patient-xenograft tumors and enhance anti-tumor activity (73) (**Figure 1E**). This strategy of using heparanase to increase the penetration of CAR-T cells into tumors shows promise to increase efficacy of the therapy.

Heparanase in DC Vaccines

Heparanase overexpression has been documented across the majority of tumor types, including solid tumors (74–77) and hematological tumors (22, 78). Thus, heparanase represents a potential tumor associated antigen (TAA) that could be exploited across multiple cancer types. Dendritic cell vaccines are a novel approach to selectively target tumor cells overexpressing TAA. Engineered dendritic cells overexpressing TAA can generate antigen-specific T cells that have increased cytotoxicity against tumor cells (79–81).

Heparanase-specific and reactive $CD8^+$ T cells were identified in the bone marrow of a sample of breast cancer patients, and were functionally reactive to heparanase-overexpressing tumor cells (82). The overexpression of heparanase in DCs isolated from PBMCs was shown to enhance the activation of T cells from matching donors, and consequent cytotoxicity against target gastric cancer cells (83). This finding also held true in an animal model, where murine DCs were pulsed with murine heparanase peptides and injected into mice. This vaccine could induce cytotoxic T lymphocytes (CTLs) in mice specific to H-2kb-expressing mouse tumor cell lines (B16, LLC, and EL-4) (84). In addition, administering heparanase peptide-pulsed DCs after injecting B16 tumor cells could slow tumor growth (84). Furthermore, the immunogenicity and efficacy of these peptides was increased when generated in the branched multiple antigenic peptide conformation (85, 86).

The heparanase peptide has also been tested as a TAA in a prophylactic vaccine. Priming mice *in vivo* with human heparanase peptides (Hpa525, Hpa277, and Hpa405) generated CTLs that specifically targeted human tumor cell lines presenting heparanase on either HLA-A*0201 (87) or HLA-A2 (88) (**Figure 1E**). Injecting heparanase-pulsed DCs into mice before

administering B16 tumor cells was shown to protect animals from tumor growth (84).

These data suggest that heparanase could be a robust tumor antigen, as when targeted, shows both reduction and protection against tumor growth in animal and human systems. However, targeting heparanase via a vaccine approach will rely on tumors maintaining heparanase expression to allow T cell recognition of heparanase-positive tumor cells. Regardless, the selective pressure from these vaccines on tumor cells to downregulate heparanase expression would still be advantageous in blocking tumor progression.

Heparanase in Viral-Therapeutic Delivery

The delivery of gene therapy as a cancer treatment requires specific targeting to tumor cells. A promising approach to target therapies toward tumors is through viral particles via attachment to T cells (89). An important step in the delivery of these therapies is the release or "hand off" (transfer of viral particles from T cells to target tumor cells), when T cells release their viral cargo at the tumor site (89). Heparanase present in the tumor microenvironment, either from malignant cells or activated T cells, was shown to promote viral "hand off" for the successful delivery of anti-tumor molecules to the tumor cells (89) (**Figure 1E**). Another study using viral gene therapy to treat a murine model of malignant plural mesothelioma found that co-infection with a heparanase-expressing adenovirus vector could enhance efficacy of virotherapy and penetrance into tumors (90), a previous limitation of this therapy. This approach showed a reduction in tumor weight, and an increase in overall survival of animals inoculated with mesothelioma. This is likely a result of heparanase increasing ECM breakdown, as heparanase was shown to enable viral particles to penetrate deeper into tumor spheroids (90).

The robust expression of heparanase across multiple cancer types and cell types makes it a useful target to manipulate and utilize its anti-cancer properties. All of these therapies described will rely on maintained heparanase expression for efficacy. As we will describe, heparanase inhibitors currently used to reduce tumor burden may not always have favorable outcomes on tumor progression, especially for patients undergoing the therapies described above.

HEPARANASE INHIBITORS AND LEUKOCYTE FUNCTION

Our understanding of the relationship between heparanase and leukocytes during tumor progression remains limited. Similarly, much is still unknown about how heparanase inhibitors affect the anti-tumor immune response, despite their current use in clinical trials against a range of cancers (91–94). A number of heparanase inhibitors in anticancer therapy have been recently reviewed (95–97). These include the heparan sulfate mimetic Roneparstat (SST0001), 2-O-,3-O-desulfated heparin (ODSH, also known as CX-01), Necuparanib (M402), PG545 (a heparan sulfate mimetic conjugated to a lipophilic cholestanol aglycone moiety, also known as Pixatimod) (21), and PI-88 (a heparan sulfate mimetic, also known as Muparfostat) (98).

Preclinical animal models show PG545 can reduce tumor and metastatic burden in several tumor models, including breast, prostate (21), liver (21), lung (21), colon (21), ovarian (94), head, and neck cancers (21, 99, 100), melanoma (21, 101), pancreatic cancer (102, 103), and colon cancer (104). Interestingly, the mechanism by which PG545 exerts its anti-tumor properties has been shown to be multifactorial. In addition to directly blocking heparanase activity (105), PG545 has been shown to reduce heparanase expression, possibly by inhibiting VEGF and FGF2 signaling (99). PG545 has also been shown to inhibit macrophage infiltration into pancreatic tumors (102), activate NK cells via DCs (106), and activate lymphocytes (100), as part of its anti-tumor activity. A phase I clinical trial against a range of advanced solid tumors showed that PG545 stimulated the innate immune response, resulting in an at least a two-fold increase of circulating plasmacytoid DCs and NK cells in majority of patients (93). It is perhaps predominantly through this mechanism of leukocyte activation that this inhibitor exerts its anti-cancer activity, rather than direct tumor cell-heparanase inhibition. Given its modest effects as a monotherapy (93), PG545 will most likely be used in combination with chemotherapy to treat advanced cancers for maximum efficacy.

Other clinically relevant heparanase inhibitors such as Roneparstat and ODSH also display immunomodulatory effects. Roneparstat, in development for the treatment of multiple myeloma, has been observed to effect macrophage polarization by inhibiting the expression of M1 related genes in LPS-stimulated U937 macrophages (107). In a mouse model of ischemia/reperfusion injury, inhibition of heparanase with Roneparstat reduced the number of infiltrating M1 macrophages in the kidney, resulting in lower levels of pro-inflammatory cytokines (107). ODSH is another heparanase inhibitor (108) which blocks multiple steps of inflammation. As described for heparin, ODSH reduces leukocyte rolling, adhesion, and accumulation (109, 110). ODSH has also been shown to inhibit neutrophil elastase and inflammation in a mouse model of neutrophil elastase-induced airway inflammation (111) and in the sputum of cystic fibrosis patients (112). In addition, ODSH also inhibits the accumulation of neutrophils in the airway after *Pseudomonas aeruginosa* infection (113) and protects against platelet factor 4-induced thrombocytopenia in chemotherapy and radiotherapy-treated animals by acting on megakaryocyte proliferation. Finally, ODSH inhibits high-mobility group box 1 (HMGB1) release from macrophages (111, 113, 114), a potent proinflammatory cytokine, and inhibits P-selectin-mediated macrophage adhesion (115).

More work needs to be done to define and understand the effects of heparanase inhibitors on cells of the immune system. Heparanase inhibitors have been used as anti-inflammatory agents, and have been shown to impair lymphocyte trafficking (116, 117) and leukocyte function (46, 47, 61, 118). It is possible that in some tumor settings, heparanase inhibitors may inhibit leukocyte function, and consequently tip the balance away from tumor clearance and in favor of tumor progression. Heparanase inhibitors may be effective against tumors in which

leukocyte-heparanase aids tumor progression, such as colorectal and pancreatic carcinoma (52), but perhaps less effective against other solid tumors which have little heparanase expression in the tumor microenvironment. Choosing the appropriate anti-cancer therapy will lie in finding the balance in particular cancer settings between inhibiting pro-tumorigenic heparanase and promoting its anti-tumorigenic effects.

CONCLUSIONS

This review describes how leukocyte-heparanase can be a double-edged sword in tumor progression; it can enhance tumor immune surveillance and tumor cell clearance, but also promote tumor survival and growth. We also discuss the potential of using heparanase in leukocyte therapies against tumors, and the effects of heparanase inhibitors on tumor progression and immunity.

We are just beginning to understand the influence of heparanase on a pro/anti-tumor immune response, and there are still many questions to answer. How do the pro/anti-tumorigenic effects of heparanase differ across different cancer types? Does the tumorigenic effect of heparanase change during cancer progression? And how does the expression or role of heparanase change during treatment regimens? Answering these questions may help guide the appropriate use of heparanase inhibitors, and the use of heparanase-assisted therapies for the treatment of cancer.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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The Emerging Roles of Heparan Sulfate 3-*O*-Sulfotransferases in Cancer

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Alteration in the expression of heparan sulfate (HS)-modifying enzymes has been frequently observed in cancer. Consequently, dysregulation of the HS biosynthetic machinery results in dramatic changes in the HS structure, thereby impacting a range of pivotal cellular processes involved in tumorigenesis and cancer progression including proliferation, migration, apoptosis, and immune escape. HS 3-*O*-sulfotransferases (HS3STs) catalyse the maturation step of glucosaminyl 3-*O*-sulfation within HS chains. Although seven HS3ST isozymes have been described in human, 3-*O*-sulfation is a rare modification and only a few biological processes have been described to be influenced by 3-*O*-sulfated HS. An aberrant expression of HS3STs has been reported in a variety of cancers. Thus, it was suggested that changes in the expression of these enzymes as a result of tumorigenesis or tumor growth may critically influence cancer cell behavior. In accordance with this assumption, a number of studies have documented the epigenetic repression of HS3ST2 and HS3ST3A in many cancers. However, the situation is not so clear, and there is accumulating evidence that HS3ST2, HS3ST3A, HS3ST3B, and HS3ST4 may also act as tumor-promoting enzymes in a number of cancer cells depending on their phenotypes and molecular signatures. In this mini-review, we focus on the recent insights regarding the abnormal expression of HS3STs in cancer and discuss the functional consequences on tumor cell behavior. In term of clinical outcome, further investigations are needed to explore the potential value of HS3STs and/or their 3-*O*-sulfated products as targets for therapeutic strategies in cancer treatment.

Keywords: heparan sulfate, sulfotransferase, cancer, epigenetic regulation, immune escape

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INTRODUCTION

Heparan sulfate (HS) is an anionic and linear polysaccharide, which is covalently attached to core proteins to form HS proteoglycans (HSPG). These molecules are present within the extracellular matrix (ECM) and at the surface of virtually all cells. While the core protein primarily determines the localization of HSPG, HS chains are involved in the binding of a large number of proteins, including growth factors, cytokines, proteases, lipoproteins, and ECM components. HS-protein interactions have multiple effects ranging from simple immobilization to protection against degradation, conformational change, stabilization of receptor-ligand complexes, or protein oligomerization (1–5). Via these interactions, HS does not only regulate physiological processes, such as in embryogenesis, angiogenesis, blood coagulation and inflammation, but are also implicated in many pathologies, including cancer, infectious diseases, and neurodegenerative disorders (6–10).

Structural determinants in HS are derived from enzymatic modifications of the glycan backbone, which is formed by polymerization of the repeat unit consisting of D-glucuronic acid (GlcUA) and *N*-acetylated D-glucosamine (GlcNAc). In the classical model of biosynthesis, the native polysaccharide is first subject to partial *N*-deacetylation/*N*-sulfation of GlcNAc residues. This modification provides the substrate for next modifications, including epimerization of some GlcUA into L-iduronic acid (IdoUA), 2-*O*-sulfation of uronic acids (mainly IdoUA), and 6-*O* and/or 3-*O*-sulfations of GlcN residues (**Figure 1**). HS-protein interactions are primarily driven by complementarity between positively charged amino acid residues in the ligand and sulfate groups in the HS sequence. However, protein binding to HS does not only rely on the overall degree of sulfation. Instead, a concept has emerged whereby optimal binding depends on the spatial arrangement of sulfate groups in given HS sequences (2, 4, 5, 11, 12).

HS3STs represent the largest family of HS-modifying enzymes, and yet the reaction of 3-*O*-sulfation is the rarest maturation step, when compared to other sulfations. Seven HS3STs have been characterized in human, for which the expression is dependent on cell type and tissue environment (**Table 1**). HS3ST-mediated 3-*O*-sulfation leads to at least two distinct forms of 3-*O*-sulfated motifs. HS3ST1 and HS3ST5 participate in the generation of anticoagulant-active HS/heparin sequences for antithrombin-III, while HS3ST2, HS3ST3A, HS3ST3B, HS3ST4, and HS3ST6 were described to provide the HS-binding motifs for the glycoprotein gD of herpes simplex virus-1 (HSV-1) (13, 14, 33–41). To date, only a few ligands are known to selectively interact with 3-*O*-sulfated motifs, whereas hundreds of HS-binding proteins have been identified. Consequently, little is known concerning the functions of 3-*O*-sulfated HS in biological processes, apart from their roles in anticoagulant properties of HS/heparin and entry of HSV-1 into host cells (12, 42, 43).

Expression of the genes encoding HS-modifying enzymes is frequently dysregulated in cancer and other diseases (42, 44, 45). An aberrant expression of HS3STs has been reported in various cancers, suggesting that these enzymes and their 3-*O*-sulfated products may be involved in tumorigenesis and cancer progression. However, these reports reveal either anti-oncogenic or tumor-promoting effects (**Table 1**), and the mechanisms and consequences of HS3ST dysregulation in cancer still remain obscure (15–23, 26–28, 32).

ANTI-ONCOGENIC PROPERTIES OF HS3STs

In cancer cells, hypermethylation of CpG islands in gene promoters has been associated with the loss of expression of some susceptible genes, including tumor suppressor genes, and genes encoding products involved in DNA repair and apoptosis (44, 46, 47). In the attempt to identify novel silenced genes in breast cancer, Miyamoto et al. (22) found that the 5' region of the *HS3ST2* gene was hypermethylated in tumor tissue but not in surrounding non-cancerous tissue. As a consequence, the expression level of HS3ST2 was markedly reduced in the cancer sample compared with the matched normal counterpart. Then, they demonstrated that HS3ST2 was not expressed in cell lines representative of the different molecular breast cancer subgroups (48). Reversing methylation restored the expression of the enzyme, confirming the silencing effect of gene methylation. Moreover, *HS3ST2* gene hypermethylation was detected in the majority of primary breast cancer samples analysed, and also in human colon, lung and pancreatic cancers (22). Following this work, many clinical studies have been published examining the relationships between aberrant methylation of the *HS3ST2* gene and tumorigenesis. Hypermethylation was found at high frequency in gastric, breast, colorectal, prostate and cervix cancers, as well as in hematological neoplasms (15–23). In breast and cervix, hypermethylation of the *HS3ST2* gene occurs early during malignant transformation, suggesting a correlation between HS3ST2 silencing and progression of the disease (16, 23). Hwang et al. (18) demonstrated that the exogenous re-expression of HS3ST2 was efficient to inhibit cell migration, invasion and proliferation in various lung cancer cell lines. However, they found that the tumor size was not significantly different between patients with *HS3ST2* gene hypermethylation and those without, in spite of the anti-proliferative property of HS3ST2 observed *in vitro*. Hence, they emphasized the need of further investigations to validate HS3ST2 silencing as a prognostic/predictive biomarker (18).

Besides HS3ST2, an analysis of the methylation status of other genes encoding HS sulfotransferases in chondrosarcoma showed hypermethylation in proximal regions of the *HS3ST1* and *HS3ST3A1* genes. Exposure to a demethylating agent restored their expression, confirming that aberrant methylation had affected their transcription. Moreover, re-expression of HS3ST3A reduced the proliferative and migratory properties of chondrosarcoma cells, suggesting that silencing of this enzyme may have contributed to tumor cell growth and invasiveness (26). In the following study, Mao et al. (27) demonstrated that the *HS3ST3A1* gene is epigenetically repressed in breast cancer cell lines representative of the different molecular subgroups, except in the human epidermal growth factor receptor 2-positive (HER2+) cell lines. Re-expression of the enzyme in luminal A-type MCF-7 and triple negative MDA-MB-231 cell lines reduced cell proliferation *in vitro* and tumor growth in xenografted mice. Thus, the authors hypothesized that modification in HS structure may have hindered the interactions of growth factors with signalling receptors (27).

TUMOR-PROMOTING ACTIVITIES OF HS3STs

Albeit that epigenetic repression of the *HS3ST2* gene was related to progression of many cancers, Vijaya Kumar et al. (24) reported that its re-expression in MDA-MB-231 cells led to an increase in cell viability and invasion. Likewise, we reported that MDA-MB-231 cells carrying HS3ST2 expression displayed a significant increase in proliferation and survival (25). The

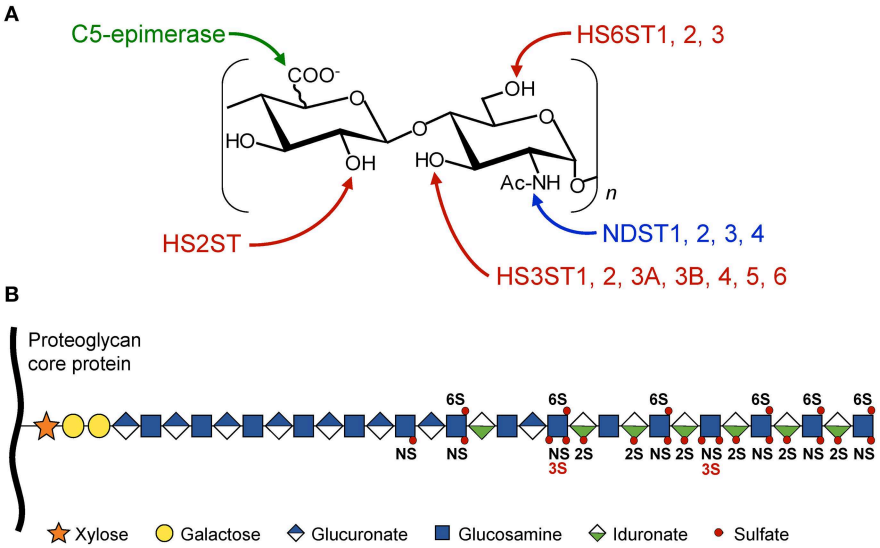

FIGURE 1 | Schematic representation of HS modifications. The HS polysaccharide is linked by the tetrasaccharide linker [Xyl-Gal-Gal-GlcUA] to a specific serine residue within the HSPG core protein. Elongation of the chain is achieved through the alternative addition of GlcUA and GlcNac residues by the polymerases EXT1/EXT2. **(A)** The disaccharide units [GlcUA-GlcNAc] are modified by the actions of sulfotransferases and epimerase. The sites of modification by *N*-deacetylases/*N*-sulfotransferases (NDST), C5-epimerase, HS 2-*O*-sulfotransferase (HS2ST), HS 6-*O*-sulfotransferases (HS6STs), and HS 3-*O*-sulfotransferases (HS3STs) are indicated. **(B)** HS modifications do not go to completion, resulting in domains with high, intermediate, and low levels of sulfation, enabling the generation of many HS structures and potential ligand-binding sites. NS, *N*-sulfo group on GlcN residue; 2S, 2-*O*-sulfo group on uronic acid residue; 3S and 6S, 3-*O* and 6-*O*-sulfo groups on GlcN residue.

TABLE 1 | Tissue expression of human HS3STs.

Enzyme	Expression in normal tissues	Potential role in cancer
HS3ST1	cerebellum (high), spleen (high), cerebral cortex, kidney, lung, stomach, small intestine, colon, testis, liver, heart, pancreas, placenta (13, 14)	/
HS3ST2	cerebral cortex (high), cerebellum, placenta, spleen, lung, stomach, small intestine, colon, testis (13, 14)	anti-oncogenic (15–23) pro-tumoral (24, 25)
HS3ST3A	liver, placenta, spleen, stomach, small intestine, colon, testis, heart, lung, kidney, pancreas (13, 14)	anti-oncogenic (26, 27); pro-tumoral (27)
HS3ST3B	liver (high), placenta (high), spleen (high), stomach, small intestine, colon, testis, skeletal muscle, heart, lung, kidney, pancreas (13, 14)	pro-tumoral (25, 28–31)
HS3ST4	cerebral cortex (high), cerebellum, stomach, spleen, testis (13, 14)	pro-tumoral (25, 32)
HS3ST5	skeletal muscle (high), placenta, cerebral cortex, cerebellum, small intestine, colon (13, 33, 34)	/
HS3ST6	liver, kidney (35)	/

pro-invasive phenotype was however not observed in the MCF-7 cell line (24), suggesting that the consequence of HS3ST expression could be different depending on the breast cancer phenotype. In line with this assumption, Mao et al. (27) described that HS3ST3A expression enhanced proliferation and survival of HER2+ SKBR3 cells, but not in MCF-7 cells. However, these authors reported that HS3ST3A was also anti-proliferative in MDA-MB-231 cells, meaning that this isozyme produced opposite effect than the one promoted by HS3ST2 in the same cell line (24, 25). Although intriguing, we also found that overexpression of HS3ST3A did not have any effects on the proliferation of MDA-MB-231 cells. In contrast, forced expression of HS3ST3B and HS3ST4 had the same functional impact as observed in the case of HS3ST2 (25). These results suggest that the impact of HS3ST expression in breast cancer cells could be also dependent on the type of isozyme. One explanation may be that each HS3ST exhibits subtle differences in their substrate requirement. On this assumption, HS3ST3A may have a restricted substrate specificity, making it incapable of synthesizing 3-*O*-sulfated HS with a tumor-promoting property in MDA-MB-231 cells. Conversely, HS3ST2, HS3ST3B, and HS3ST4 may exhibit a broader selectivity or share, at least in part, some common acceptors.

In pancreatic cancer cells, high level expression of HS3ST3B was reported to induce epithelial-mesenchymal transition (EMT) and to enhance cell invasiveness *in vitro*. Moreover, HS3ST3B overexpression was associated with an increased angiogenesis in graft-bearing mice, supporting the idea that HS3ST3B could favor pancreatic cancer progression (29). In the continuity of this study, Zhang et al. (30) reported that high expression of HS3ST3B in U937 leukemia cells enhanced cell proliferation and survival, while its silencing had opposite effects. The advantage given by HS3ST3B was related to an increase in the production of vascular endothelial growth factor (VEGF) and activation of downstream signalling pathways. Next, the authors demonstrated that conditioned medium of HS3ST3B-expressing

U937 cells had a promoting effect on angiogenesis, which was dependent on the secretion of VEGF. Finally, they confirmed that HS3ST3B effectively promoted leukemia cell proliferation and VEGF-dependent angiogenesis in xenografted mice (30). Most recently, a clinical study conducted in a cohort of lung cancer patients uncovered that HS3ST3B expression was upregulated in tumor biopsies compared to that in matched normal tissues (28). A high level expression of the enzyme was also observed in NSCLC cell lines. Silencing its expression reversed the mesenchymal phenotype, meaning that HS3ST3B is involved in the regulation of EMT in lung cancer cells in the same way as in pancreatic cells (28, 29). High expression level of TRF2 (telomere repeat binding factor 2), a protein normally involved in telomere protection, has been observed in various human cancers. Interestingly, the *HS3ST4* gene was identified as a transcriptional target of TRF2, and increasing TRF2 level led to an up-regulation of *HS3ST4* gene expression. Moreover, exogenous expression of either TRF2 or HS3ST4 in various tumor cell lines similarly resulted in increased tumor growth in xenografted mice, which suggests that the expression of this enzyme may be part of a pro-oncogenic pathway (32).

HS3STs AND MODULATION OF SIGNALLING PATHWAYS

Consistent with a pro-invasive phenotype, Erk1/2 and β-catenin signalling was upregulated in HS3ST2-expressing cells in an HS-dependent manner (24). As a consequence, the expression of several target genes involved in cancer cell invasiveness and survival was increased. High level expression of HS3ST3B in U937 leukemia cells was associated with activation of Notch-1, Erk1/2 and Akt signalling (30), and more recently, the tumor-promoting effects of HS3ST2, HS3ST3B, and HS3ST4 were related to sustained activation of Src, Akt, and NF-κB, and up-regulation of the anti-apoptotic proteins survivin and XIAP (25). Importantly, all these signalling molecules have been well described to play a critical role in promoting tumor growth and resistance to apoptosis (49, 50).

Most of the studies conducted with cancer cell lines reported that HS3ST overexpression resulted in an increase in the level of 3-*O*-sulfated motifs (24, 25, 27). Consequently, 3-*O*-sulfation may have influenced ligand binding to cell surface HS, leading to an alteration of diverse signalling processes. Whether 3-*O*-sulfation can modulate ligand-receptor interactions was however unknown, until neuropilin-1 (Nrp1) was described as a preferential ligand for 3-*O*-sulfated HS (43). Initially described as a co-receptor for VEGFs and class 3 semaphorins in endothelial cells and neurons, there is now evidence that Nrp1 is also expressed in a number of cancer cells, wherein it regulates cell growth, migration, invasion, and immune escape, by interacting with a broad spectrum of growth factors (51–53). Importantly, HS was reported to contribute to formation of a high-affinity complex incorporating Nrp1, VEGF, and cognate signalling receptors (54, 55). Zhang et al. (30) described that the tumor-promoting effect of HS3ST3B in leukemia cells was dependent on an autocrine activation of VEGF-dependent signalling pathways. Thus, it may be suggested that 3-*O*-sulfation of HS has improved interplay between Nrp1, VEGF, and its receptors. Besides VEGF, transforming growth factor (TGF)-β has been also identified as a ligand of Nrp1. Interestingly, HS3ST3B was described as a regulator of TGF-β-mediated EMT in NSCLC cells (28). Though not mentioned in the study, these findings suggest a possible participation of Nrp1 and 3-*O*-sulfated HS in the response induced by TGF-β. Along the same lines, we demonstrated that silencing of Nrp1 in MDA-MB-231 cells reversed the advantage given by HS3ST3B (31). Hence, these findings raise the possibility that the tumor-promoting properties of HS3ST3B could be dependent on the formation of signalling complexes containing Nrp1.

Besides the roles attributed to HS moieties, HSPG core proteins have binding properties that engage them in specific interactions with proteins involved in signalling and cytoskeleton organization (1, 3). A number of studies have reported that the expression of HSPG is dysregulated in many cancers, thus altering key biological processes involved in cell proliferation and survival (9). However, there is no evidence that changes in the expression of a core protein can alter the sulfation patterns within HS chains (56). Recently, Corti et al. (57) reported that HS chains of syndecan-2 contained higher levels of 6-*O* and 3-*O* sulfations, which was related to an increase in formation of a signalling complex between syndecan-2, VEGF and its receptor in endothelial cells. These last findings demonstrate the existence of a regulatory mechanism wherein a core protein determines the sulfation pattern of its own HS chains. High level expression of syndecan-2 has been observed in many cancers (9). This suggests that such a regulatory mechanism of HS sulfation may also occur in cancer cells. This could lead to the appearance of HSPG with specific HS chains, thereby enhancing the binding and functions of certain HS ligands. This assumption deserves additional works to identify the HSPG and their relevant ligands that interact with 3-*O*-sulfated HS in cancer cells.

HS3STs AND ESCAPE TO IMMUNE SURVEILLANCE

A body of evidence has accumulated over the past two decades indicating that HS3ST2 is epigenetically silenced in a wide range of cancers and tumor cell lines (15–23). However, the authors did not address the possibility that another HS3ST could be expressed in place of HS3ST2. This assumption is supported by clinical studies showing that HS3ST3A and HS3ST3B were highly expressed in biopsies from patients with HER2+ breast cancer (27) and lung cancer biopsies (28), respectively. These observations suggest that HS3STs can compensate each other for loss of their expression depending on the molecular signature and tissue environment of cancer cells.

During cancer progression, developing tumor cells are exposed to pro-inflammatory mediators that enhance immune anti-tumoral response. In order to evade this immune pressure, tumor cells can change their intrinsic features, thereby resulting in the emergence of cellular variants with increased activation of pro-oncogenic pathways, and less immunogenic phenotype

(58). It is of note that upregulation of the expression of HS3ST3B has been observed in many cell types exposed to inflammatory stimuli (28, 59–62). On the other hand, a progressive upregulation of TRF2 was observed during progression of colon cancer. Increased expression of TRF2 was associated with an abnormal expression of HS3ST4, which in turn led to inhibition of NK cell activation and recruitment. The same effects were observed with cancer cells carrying an exogenous expression of HS3ST4, suggesting that the isozyme may be involved in a mechanism of immune escape (32). In line with these findings, we reported that HS3ST-transfected MDA-MB-231 cells were more resistant to apoptosis induced by death receptors ligands or NK cells *in vitro* (25). The functions of certain NK cell receptors can be modulated through interactions in cis with HS on NK cells themselves or in trans with HS on target cells. Disruption of cis-interactions releases NK receptors and enhances NK cell functional response. Interestingly, silencing of HS3ST3B in NK cells was found to down-regulate the cis-interactions between HS and the NK receptors KIR2DL4 and NKp46, meaning that the functions of these receptors can be regulated through interactions with 3-*O*-sulfated HS (63, 64). It is thus tempting to speculate that 3-*O*-sulfation in cancer cells may allow cell surface HS to engage in trans interactions with NK cell receptors. Accordingly, upregulation of the expression of certain HS3STs, such as HS3ST3B or HS3ST4, may be a mechanism that permits cancer cells to impact NK cell activation and to escape their elimination. On that assumption, the tumor-promoting properties of HS3STs may rely not only on alteration of intrinsic processes in cancer cells but also on a non-cell autonomous mechanism bypassing immune surveillance.

CONCLUSIONS AND PROSPECTS

The regulation of HS biosynthesis is still poorly understood, and whether other factors can influence specific HS sulfation in a given cell type remains largely unknown. In this respect, we demonstrated that HS3ST3B is a Golgi-resident enzyme, while HS3ST2 is specifically addressed to the plasma membrane. This suggests that different subcellular location of HS3STs may be a regulatory mechanism to produce distinct 3-*O*-sulfated motifs (65). Initially, HS3STs have been divided into two groups, based on their contribution to the synthesis of anticoagulant-active sequences and binding motifs for HSV-1 gD protein. However, there is recent evidence that the situation is not so simple, and a better characterization of the catalytic activity of HS3STs and its regulation is required to define more precisely the biological functions of each isozyme (40, 42, 66, 67). It is also of note that most of the effects attributed to HS3STs in cancer are arising from *in vitro* experiments. These findings need to be regarded with caution, because interference with other metabolic processes can have a dramatic impact on cell behavior, without being linked necessarily to changes in HS sulfation (68, 69). All of this suggests that alteration in the expression of HS3STs in cancer cells may have diverse functional impacts, which could explain the different action of a particular isoform in a given cell type. Accordingly, the roles of HS3STs in cancer need to be further explored to evaluate the potential of these enzymes as targets for therapeutic strategies in cancer treatment.

Up to now, a lot of attention has been focused on HS mimetics (6, 70). A typical example is the synthesis of the pentasaccharide that binds antithrombin III (71). However, it is still difficult to synthesize oligosaccharides with complex sulfation patterns. A seducing alternative is the use of a chemo-enzymatic approach, in which controlled sulfation could be achieved by recombinant enzymes (42, 72, 73). To date, most of the HS3STs have been cloned and used to prepare 3-*O*-sulfated oligosaccharides (13, 40, 41, 43). Some of them have proven to be effective as anticoagulant agents (74) and inhibitors of HSV-1 entry (75). On the other hand, targeting HS3STs directly to hinder the reaction of 3-*O*-sulfation may be a challenging endeavor. Byrne et al. (76) reported that HS2ST was a target for a variety of cell-permeable small molecules, including kinase inhibitors. These findings suggest that such molecules could be redesigned for specific inhibition of HS sulfotransferases. On this assumption, designing specific HS3ST inhibitors via high-throughput screening of bio-active agents might be a future strategy to control HS 3-*O*-sulfation in cancer cells. In conclusion, a better understanding of the functions of HS3STs in cancer cells may provide opportunities to use these HS-modifying enzymes as molecular targets to improve therapeutic strategies.

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AD and FA: conception, design, writing, and review of the manuscript.

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The Expression, Regulation, and Biomarker Potential of Glypican-1 in Cancer

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Glypican-1 (GPC-1) and other glypicans are a family of heparan sulfate proteoglycans. These proteins are highly expressed on the cell membrane and in the extracellular matrix, functioning mainly as modulators of growth factor signaling. Some of them are abnormally expressed in cancer, possibly involved in tumorigenesis, and detectable in blood as potential clinical biomarkers. GPC-1 is another glypican member that has been found to be associated with some cancers, and has increasingly interested the cancer field. Here we provide a brief review about GPC-1 in its expression, signaling and potential as a cancer biomarker.

Keywords: heparan sulfate proteoglycan, glypican, GPC-1, cancer, biomarker

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INTRODUCTION

As a second leading cause of human death, cancer still remains a major health problem in the world (1). Research has revealed major oncogenic signaling pathways, including cell cycle, histone modification, apoptosis, and other biological processes and cellular pathways (2, 3). Although these are essentially important in understanding of the cancer development, most of these pathway components locate intracellularly, making them neither efficiently accessible therapeutic targets, nor ideal for clinical biomarker discovery.

The roles of the extracellular cues have been increasingly recognized in cancer development, in which they can significantly modulate the hallmarks of cancer (4–6). Heparan sulfate proteoglycans (HSPGs) which are mainly at the cell surface and in the extracellular matrix, have gained considerable scientific interest (7–9). They become a new research topic in the cancer field (10, 11).

Glypicans are one of the HSPG families. These membrane-bound proteins participate in organ development by modulating extracellular growth signals and morphogen gradient formation, and are involved in human overgrowth and skeletal dysplasia problems (12). In some cancers, they are highly expressed, associated with tumorigenesis, and regulating angiogenesis for cancer progression and invasion (13, 14). Their causative role in tumorigenesis is supported by genetic evidence (15).

Like other glypicans, Glypican-1 (GPC-1) is recently found to be overexpressed in certain cancers, and involved in the tumorigenesis of certain cancers (16, 17). Importantly, some studies reported its level was increased in the peripheral blood of patients, holding a great promise as a new glypican biomarker in the cancer field (8, 18).

HEPARAN SULFATE PROTEOGLYCANS, GLYPICANS, AND GPC-1

The HSPGs are glycosylated proteins composed of a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. GAGs are linear tandem repeats of disaccharide units that consists of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) together with an uronic sugar (glucuronic acid or iduronic acid) or a galactose. Currently, six GAGs have been found: heparin (HP) and heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA), with different amino and uronic sugars in their disaccharide units (19). Except HP and HA that are secreted in free forms without covalent attachment to any proteins, the other four GAGs are bound to a core protein at the Ser residue of a Ser-Gly dipeptide sequence to form a proteoglycan (20). HSPGs are widely present on cell membranes and in extracellular matrices, depending on the structure and the tissue expression of their core proteins. HSPGs are usually divided into three major classes: the glycerophosphatidylinositide (GPI)-anchored type which is at the surface of the membrane (such as glypicans), the transmembrane type (such as syndecans), and the extracellular matrix type (such as agrin and perlecan) (21). HSPGs act as co-receptors for signal transduction, playing important roles in cell growth, immune response, and tumorigenesis, etc. (10, 22, 23).

Glypicans are one of the HSPG families, including glypican-1 (GPC-1) through−6 (GPC-6) in mammals with the main difference in the number of the HS chains and the protein attaching site. These proteins are located on the cell membrane, anchored by glycosylphosphatidylinositol (GPI) which is cleavable by the lipase Notum (24). Glypicans are crucial for cancer cell growth, metastasis, and angiogenesis of many human cancer cell types (13, 15). Abnormal expression of glypicans has been noted in multiple types of cancer. For examples, GPC-3 is closely related to the occurrence and development of tumors, such as human hepatocellular carcinoma, ovarian cancer and melanoma (25–27). GPC-2 is associated with neuroblastoma (28, 29).

GPC-1 is composed of a protein (558 amino acids) with the attachment of three HS chains at S486, S488, and S490, respectively. It has both a membrane-anchored form (by GPI at S530) and a secreted soluble form (30). It can also be cleaved by Notum (14, 31). GPC-1 is mainly expressed in the central nervous system and the skeletal system during embryonic development, and is expressed in most tissues in adults (32). Like other HSPGs and glypicans, GPC-1 functions through binding of growth factors, cytokines, enzymes, viral proteins, and other factors by its HS side chains. It is involved in neurodegeneration and cancer development (33–36).

GPC-1 EXPRESSION IN CANCER

Studies have shown that GPC-1 is abnormally expressed in a variety of tumor tissues and is associated with the cancer development. Earlier studies employed northern blot and immunohistochemistry, and found both GPC-1 mRNA and protein expression levels were elevated in the pancreas with cancer, compared to normal controls and the pancreas with chronic pancreatitis (37). This was further confirmed by Kayed et al. who used quantitative PCR, and GPC-1 was demonstrated to be mainly localized in pancreatic cancer cells and adjacent fibroblasts (38). Moreover, the GPC-1 expression was significantly correlated with pathologic grades and clinical stages of the pancreatic cancer, and closely associated with the poor prognosis of patients (39).

Increased expression of GPC-1, but not of other glypicans, was also detected in cultured pancreatic cancer cell lines (16). In this study, knockdown of GPC-1 expression in cells inhibited the mitotic response to fibroblast growth factor−2 (FGF-2), suggesting that GPC-1 might play an important role in the initiation and progression of pancreatic cancer.

GPC-1 expression was also increased in breast cancer tissues (17), ovarian malignant tumors (40), prostate cancerous epithelial cells (41). Moreover, 98.8% of esophageal cancer tissues demonstrated an overexpression of GPC-1 and its association with a poor prognosis (42). However, the expression of GPC-1 in colorectal cancer was controversial. Fernández-Vega et al. reported that both GPC-1 mRNA and protein expression levels were increased in colorectal cancer (43), while De Robertis et al. found the GPC-1 mRNA was decreased in metastatic colorectal cancer and non-metastasis colorectal cancer tissues (44).

Possible mechanisms of GPC-1 expression in cancer might involve microRNA expression and DNA hypomethylation. Normally, microRNA-96-5p and microRNA-149 bind to the 3′-UTR region of GPC-1 transcript to suppress its expression. However, the expression of these two microRNAs is often reduced in the pancreatic cancer (45). In addition, two important regulatory molecules, KRAS and ecotropic viral integration Site 1 (EVI1), are two known drivers of the pancreatic carcinogenesis. They both can upregulate GPC-1 expression, in which EVI1 suppresses the microRNA-96 expression (46). Another important mechanism is about the promoter hypomethylation occurring in the GPC-1 gene in the pancreatic ductal adenocarcinoma, in which the GPC-1 mRNA and protein levels are found to be significantly increased (16).

GPC-1 SIGNALING IN CANCER

Glypicans mediate signaling in cell proliferation, differentiation, and organ development, by interacting with cell membrane receptors via its HS side chains, including Wnt/β-catenin, Hedgehog (Hh), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β), etc (13, 15). The mode of action of GPC-1 is well exemplified in the FGF-2 signaling pathway. By binding to the HS chains of GPC-1, the FGF-2 and its receptor FGFR are more efficiently assembled and stabilized, and the ligand FGF-2 is protected from degradation. Besides, the participation of GPC-1 in the assembly also facilitates the dimerization of the FGFR, leading to the accelerated self-phosphorylation that initiates the signal transduction in protein

kinase B (PKB), mitogen-activated protein kinase (MAPK) and other cellular signaling pathways (47, 48).

The altered cellular activities and biological processes induced by GPC-1 might be through the modulation of the FGF-2 signaling, the well-known pathway in the regulation of cell growth, survival, differentiation, and neovascularization, tumorigenesis (49, 50). Qiao et al. showed that GPC-1 expression enhanced the growth of brain endothelial cells and sensitized them to mitogenesis induced by FGF-2. Overexpression of glypican-1 resulted in increased angiogenesis and radiation resistance in brain gliomas (51). Interestingly, GPC-1 increased the level of microRNA-149 through activation of FGFR1, and this microRNA in turn repressed other FGFR1 downstream regulations. This negative feedback loop decreased the endothelial cell response to the angiogenic stimulus of FGF (52). Although GPC-1 is positively involved in the FGFR signaling, this effect might be counteracted by its soluble form secreted in the extracellular space (23).

GPC-1 not only regulates FGF-2, but also modulates the VEGF-A signaling. VEGF is a key factor for angiogenesis, one of the essential biological processes for tumorigenesis (53). Both VEGF-A and FGF-2 are a type of heparin binding growth factors (HBGFs) whose signaling strength and duration might be tuned by GPC-1 (54). Both of their signaling were inhibited after GPC-1 was knocked down in a mouse model of pancreatic cancer (55). Moreover, hepatic endothelial cells isolated from mice lacking GPC-1 demonstrated an attenuated mitogenic response to VEGF-A (56).

In addition, the GPC-1 also modulates the TGF-β signaling pathway (**Figure 1**). TGF-β signaling pathway is involved in tumor initiation and progression by regulating cell proliferation, angiogenesis, cancer cell stemness, epithelial mesenchymal transition, invasion and inflammation (57). Here GPC-1 also interacts with the ligand and the receptor to promote the TGF-β signaling. Reduced GPC-1 expression attenuated the TGF-β1 induced inhibition of cell growth, with suppressed Smad2 phosphorylation, and plasminogen activator inhibitor-1(PAI-1) promoter activity in pancreatic cancer cells (58). Kayed et al. analyzed more thoroughly the role of GPC-1 in the TGF-β signaling, in which they found GPC-1 reduction led to a shifted response toward TGF-β, activin-A and the bone morphogenetic protein-2 (BMP-2) upon p21 induction and Smad2 phosphorylation, resulting in inhibited pancreatic cancer cell growth (38).

GPC-1 AS A CLINICAL BIOMARKER FOR CANCER

As the GPC-1 anchored on cell membrane is cleavable and it also has a secreted soluble form, it is detectable in the peripheral blood system, prompting extensive studies carried out on its potential as a clinical biomarker. In 2015, from the 48 proteins identified in the exosomes derived from the cancerous tissue by mass spectrometry and bioinformatics analysis, Melo et al. first reported that GPC-1 could be used as a marker of pancreatic cancer. Subsequently, detection of GPC-1 in human serum

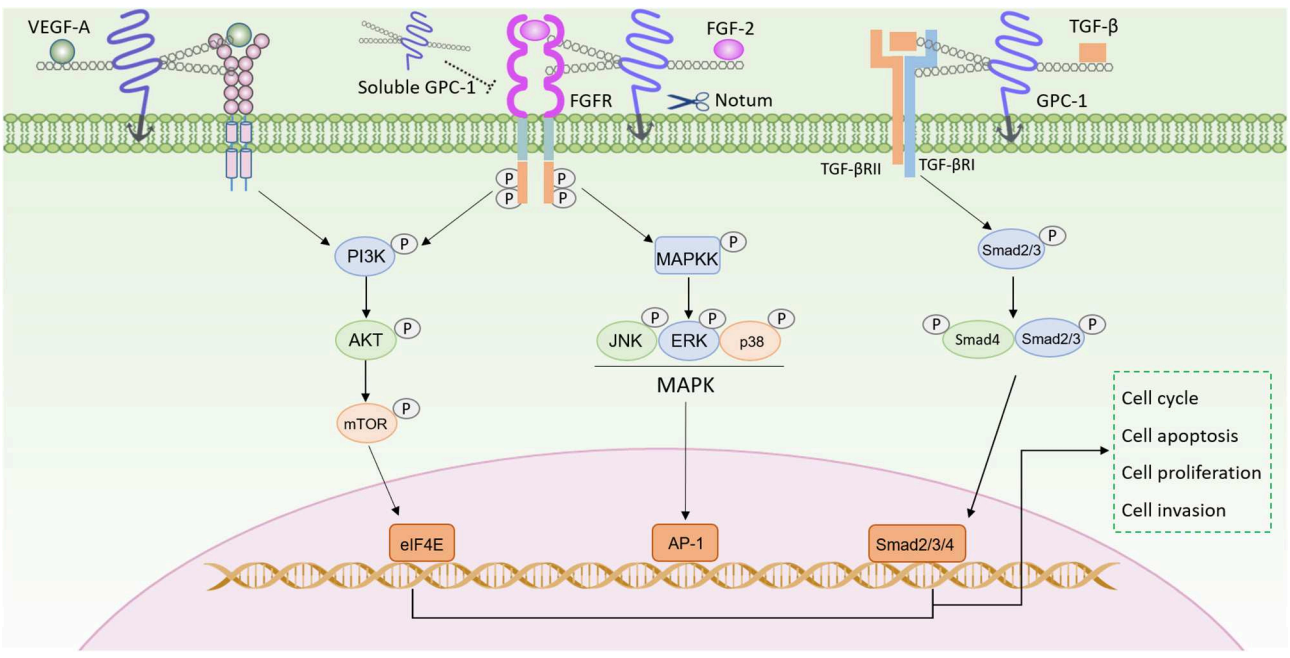

FIGURE 1 | GPC-1 modulates signaling pathways in cancer progression. The HS side chains of GPC-1 bind both growth factors (such as VEGF-A, FGF-2, and TGF-β) and their receptors, to facilitate their assembly for enhanced signaling in PI3K/AKT, MAPK, Smad pathways. GPC-1 can be cleaved by Notum and then released into the extracellular space, which can compete with the GPC-1 anchored on the cell membrane to inhibit its function.

TABLE 1 | Circulating GPC-1 as a diagnostic and prognostic marker for cancer.

Study (Reference)	Cancer type	Country	Case #	Sample type	Sample preparation	Detection method	Antibody	Results
Melo et al. (59)	Pancreas cancer	USA	190	Serum	Isolation exosomes using ultracentrifugation	Flow cytometry	PIPA528055, Thermo-Scientific	GPC-1$^+$ exosomes (from PDAC, BPD patients and healthy individuals) revealed a near perfect classifier with an AUC of 1.0 (95% CI: 0.956 – 1.0) a sensitivity of 100%.
Lai et al. (65)	Pancreas cancer	USA	29	Plasma	Isolation exosomes using ultracentrifugation	LC-MS	-	Exosomal GPC-1 is not diagnostic for PDAC, whereas a group of microRNA in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer.
Frampton et al. (66)	Pancreatic cancer	UK	27	Plasma	Isolation exosomes using ultracentrifugation	ELISA	E9038h, 2BScientific Ltd	There was no significant difference in GPC-1 levels between normal pancreas and PDAC tissues
Qian et al. (60)	Pancreatic cancer	China	28	Plasma	Isolation EVs using exoRNeasy Serum/Plasma Maxi Kit	Flow cytometry	GPC-1 antibody, GeneTex, Inc	Compared with healthy individuals, the levels of GPC-1$^+$ EVs were significantly increased in patients with advanced pancreatic cancer.
Lewis et al. (18)	Pancreatic cancer	USA	20	Whole blood, serum, or plasma	Analysis of the biomarkers glypican-1 and CD63 were then performed directly on the chip.	ACE Immunoassay		Twenty PDAC patient samples could be distinguished from eleven healthy subjects with 99% sensitivity and 82% specificity.
Yang et al. (61)	Pancreatic cancer	USA	46	Plasma	Isolation exosomes using ultracentrifugation	Nanoplasmonic sensors	BAF4519, R&D Systems. PA524972, Thermo Fisher.	GPC-1 alone, had a sensitivity of 82% (CI, 60 to 95%) and a specificity of 52% (CI, 30 to 74%) for PDAC detection.
Li et al. (45)	Colorectal cancer	China	102	Plasma	Isolation exosomes using ExoCapTM Kit	Flow cytometry	Anti-GPC-1 antibody, Santa Cruz.	The percentage of plasma GPC-1$^+$ exosome was significantly higher in CRC patients before surgical treatment than that in healthy controls and in CRC patients after surgical therapy.
Campbell et al. (63)	Prostate cancer	Australia	41	Urine	Urine cell sediments	Immunofluorescence assay	Monoclonal antibody, MIL-38	Discriminated between prostate cancer and BPH urine specimens with a sensitivity and specificity of 71% and 76%.
Levin et al. (64)	Prostate cancer	Australia	15	Plasma serum	Plasma and serum sample	Luminex assay	Monoclonal antibody, 3G5	Circulating GPC-1 was reduced in prostate cancer patients vs. non-prostate cancer patients.
Lucien et al. (67)	Pancreatic cancer	USA	93	Plasma	Detecting extracellular vesicles based on calibration beads	Nanoscale flow cytometry	PA5-24972, Thermo Fisher.	GPC-1 was unable to discern pancreatic cancer from BPD
Zhou et al. (68)	Pancreatic cancer	China	156	Serum	Serum sample	ELISA	RayBiotech, ELH-GPC-1	The serum GPC-1 cannot be used as a serum diagnostic biomarker for PDAC patients, high levels of serum GPC-1 predict poor prognosis in PDAC patients.

exosomes was reported. In breast cancer patients, 75% had higher GPC-1^+ exosomes than the healthy controls. In pancreatic ductal adenocarcinoma (PDAC), all 190 patient serum samples had higher GPC-1^+ exosomes than healthy individuals, exhibiting a nearly perfect diagnostic value (∼100 and ∼100% in the receiver operating characteristic curve). By the Cox multivariate regression analysis, this study also reported the serum GPC-1 exosomes was an independent prognostic marker for disease-specific survival (59).

There are also other reports that employed various methodologies to evaluate the diagnostic potential of GPC-1 in cancers. Qian et al. isolated the serum extracellular vesicles (EVs) and found that the GPC-1^+ EVs was significantly higher in patients with advanced pancreatic cancer than those in healthy controls (60). Lewis et al. developed an affinity capture elution immunoassay to detect the exosomal GPC-1, which distinguished 20 PDAC patient samples from 11 healthy subjects, with 99% sensitivity and 82% specificity (18). Yang et al. used an advanced multiplexed plasmonic assay and identified a signature of GPC-1 and other four markers for PDAC detection, in which the diagnostic sensitivity and specificity of GPC-1 alone reached 82 and 52%, respectively (61).

The biomarker potential of circulating GPC-1 was also studied in other cancers. The percentage of plasma GPC-1^+ exosomes significantly increased in colorectal cancer patients than those in healthy controls, and reduced after surgical removal (62). In the urinary sediment samples from 125 patients with prostate cancer and a group of healthy individuals, the sensitivity and specificity of GPC-1 achieved 71 and 76%, respectively (63). Levin et al. also measured GPC-1 in plasma and serum samples and found it was significantly increased in prostate cancer patients as compared to the health cohorts (64). Taken together, these reports suggest that GPC-1 might be a useful marker for the diagnosis of prostate cancer. All these studies about the circulating GPC-1 as a clinical cancer biomarker were summarized in **Table 1**.

Nevertheless, there are also some studies that yielded controversial results. In a report by Zhou et al. serum GPC-1 level was concluded to be a prognosis factor but not an ideal marker for the clinical diagnosis of PDAC (68). Similar finding was reported by Frampton Prado et al. (66). Lai et al. found the plasma exosomal GPC- 1 level could not differentiate the PDAC patients from the controls, while a panel of microRNAs in the exosomes was a superior pancreatic cancer biomarker instead (65). Moreover, Lucien et al. measured the GPC-1^+ EVs in the blood samples, and found they were not able to separate the pancreatic cancer patients from those with benign pancreatic disease effectively (67).

There are numerous reasons that might account for these controversial results. First, GPC-1 is not a tissue-specific protein. The human protein atlas database (https://www.proteinatlas.org/) and the comprehensive human tissue proteome analysis that GPC-1 is widely expressed in brain, gastrointestinal tract, urinary, and reproductive systems (69). GPC-1 expressed from the cancerous tissue is probably confounded by these normal secretions from other tissues. Next, in many studies mentioned above, the specificity of the GPC-1 antibody was not seriously validated, which could easily generate false results (70). Many of these antibodies were generated by synthetic short peptides or protein fragments expressed in non-mammalian systems, thus they lacked necessary modifications (especially glycosylations and HS chains on GPC-1) and genuine structures. Ideally, the immunohistochemical staining of GPC-1 should be validated by Western blots with the same antibody, to show whether the blots demonstrated any other non-specific bands and whether the results in these two methods were well correlated. In addition, few studies had thoroughly examined the relationship between the serum GPC-1 levels and the cancer tissue size, the percentage of GPC-1^+ cells, and the GPC-1 concentration of the total cancer tissue homogenate. In addition, the release of GPC-1 relies on the protease Notum which might not always be expressed in normal amount and activity in cancerous tissues. Notably, many studies used serum as the sample for GPC-1 measurement. The serum differs from the plasma not just in the missing of fibrinogen and other components, but importantly, contains a tremendous amount of active clotting factors, each of which is a highly active protease. It is not known whether any of them might cleave GPC-1, leading to false results. Some studies used the EVs of particular sizes from the plasma for the analysis. However, it remains questionable whether these EVs represent the entire EVs in GPC-1 expression unbiasedly. Besides, the EV extraction for GPC-1 measurements has neither yet proven to be necessary, nor feasible in clinical laboratories. Therefore, more thorough and stringent studies are expected to establish whether GPC-1 in the blood can be a clinical biomarker for certain cancers.

CONCLUDING REMARKS

Glypicans and other HSPGs are very important in the modulation of growth factor signaling. They expressed abnormally in some cancerous tissues, and causatively involved in tumorigenesis. GPC-1 is a new glypican member that has extensively been demonstrated to be increased in certain cancers. Despite a few controversial results, the biomarker potential of GPC-1 deserves further investigation. As membrane and extracellular proteins are more therapeutically accessible and bear more potential to be clinical biomarkers, GPC-1 and other HSPGs will continue to interest the research field for better elucidation of their mechanistic roles and diagnostic values in clinical settings.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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The Role of Glypican-3 in Regulating Wnt, YAP, and Hedgehog in Liver Cancer

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Glypican-3 (GPC3) is a cell-surface glycoprotein consisting of heparan sulfate glycosaminoglycan chains and an inner protein core. It has important functions in cellular signaling including cell growth, embryogenesis, and differentiation. GPC3 has been linked to hepatocellular carcinoma and a few other cancers, however, the mechanistic role of GPC3 in cancer development remains elusive. Recent breakthroughs including the structural modeling of GPC3 and GPC3–Wnt complexes represent important steps toward deciphering the molecular mechanism of action for GPC3 and how it may regulate cancer signaling and tumor growth. A full understanding of the molecular basis of GPC3-mediated signaling requires elucidation of the dynamics of partner receptors, transducer complexes, and downstream players. Herein, we summarize current insights into the role of GPC3 in regulating cancer development through Wnt and other signaling pathways, including YAP and hedgehog cascades. We also highlight the growing body of work which underlies deciphering how GPC3 is a key player in liver oncogenesis.

Keywords: Wnt signaling, heparan sulfate proteoglycan, antibody therapy, liver cancer, YAP signaling

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INTRODUCTION

Each year hepatocellular carcinoma (HCC) affects 750,000–1 million people worldwide and is projected to be the third most common cause of cancer death in the United States by 2030 (1). Glypican-3 (GPC3), a broadly conserved cell-surface proteoglycan, contains heparan sulfate (HS) chains connected to a core protein. Glypican regulation is linked to cell growth, differentiation and motility. GPC3 is highly expressed in >70% of HCCs but not in normal adult tissues (2). GPC3 has also been associated with poor prognosis in HCC patients (3). Taken together, this designates GPC3 as an established biomarker and indication of progression for HCC, a lethal disease for which there are limited treatment options (4, 5). While GPC3 is most notably studied in HCC, it has been implicated in other solid tumors as well (6, 7).

Wnt signaling is vital in embryonic development and tissue homeostasis (8). In adults, Wnt signaling promotes tissue renewal and regeneration. During the embryonic stage, GPC3 is widely expressed in a stage and tissue specific manner (9). GPC3 expression can be detected in the placenta and other embryonic tissues including the ovary, mammary, and lung. Several studies have shown that expression of GPC3 regulating tumor proliferation and progression through Wnt signaling cascades (10). Given that Wnt is highly hydrophobic and may require HS fragments functioning as a transporter or nano-storage unit to facilitate its activation in the extracellular microenvironment, the link between cell surface glypicans and Wnt would be highly interesting (11–14).

In this review, we highlight the role of GPC3 in cellular signaling including Wnt and other signaling pathways such as YAP (Yes associated protein), and Hedgehog (Hh) within the current scientific milieu. Due to the importance of GPC3 in multiple signaling cascades, GPC3 could have a pivotal role as a biomarker and as a potent therapeutic target in investigational immunotherapies (15). Therapeutics targeting GPC3 are in preclinical development and rigorous mechanistic insight could be pivotal in further developing a successful therapeutic strategy (15, 16).

BIOLOGY AND STRUCTURE OF GPC3

Glypicans, classified among the heparan sulfate proteoglycan family, reside on the exterior cell membrane via a glycosylphosphatidylinositol (GPI) anchor and are a major part of the extracellular matrix (ECM) mediating cell-ECM and cell-cell interactions (4, 17). Glypicans comprise of a core protein attached to two HS glycosaminoglycan polysaccharide chains. The structure of glypicans is evolutionarily well-conserved and the family consists of six subtypes including GPC1-6 in mammals (18). Glypicans are typically between 60 and 70 kDa and contain a secretory signal peptide at the N-terminal and a GPI anchor at the C-terminal. All glypicans have 14 conserved cysteines, which form intramolecular disulfide bridges to connect the N terminus and C terminus, even after possible furin cleavage (19). The unique structure of glypicans provides glypicans the unique capability to store and sequester various molecules including: cytokines, morphogens, chemokines, and growth factors (15). Glypicans attract these molecules and develop concentration gradients around the ECM and cellular membrane allowing for recognition of receptors with different thresholds.

During early development, GPC3 is found in the fetal organs including: liver, lung, placenta, and kidney. In most adult tissues, GPC3 is absent or lowly expressed in most adult tissues (20). Simpson Golabi Behmel syndrome (SGBS), an X-linked overgrowth disorder characterized by a broad spectrum of clinical manifestations, is due to GPC3 loss of function mutations and primarily affects males. In SGBS patients, developmental abnormalities described include enlarged tongue, polydactyly, syndactyly, cleft palate, congenital heart defects, cystic kidneys, and vertebral fusions (21). This overgrowth phenotype has also been observed in GPC3-null mice, which expire at birth in the C57BL/6 background and share several clinical abnormalities with SGBS patients (22, 23). GPC3 is located on the X chromosome (Xq26) with Isoform 2 (GenBank Accession No.: NP_004475) being the most commonly expressed. A total of four alternatively spliced variants are documented (4, 19). The functional relevance and specificity of these isoforms is unknown. GPC3 HS chains have been shown to bind molecules including Wnt (24, 25). Interestingly, studies have suggested that the GPC3 core protein may also participate in binding Wnt as a co-receptor (26, 27). Using computational structure modeling, our group recently identified a cysteine-rich domain on the N-lobe of GPC3 for Wnt functional binding, providing evidence that GPC3 is a Wnt co-receptor that modulate Wnt/β-catenin signaling in HCC cells (28). By attracting and storing growth factors via HS chains and recognizing Wnt as a co-receptor, GPC3 acts as a cell surface glycoprotein that can modulate Wnt signaling in liver cancer.

Abbreviations: HCC, Hepatocellular Carcinoma; HS, Heparan Sulfate; YAP, Yes Associated Protein; Hh, Hedgehog; ECM, Extracellular Matrix; SGBS, Simpson Golabi Behmel syndrome; FZD, Frizzled receptor; GPCR, G Protein Coupled Receptor; LRP5/6 lipoprotein receptor-related protein; DVL, Disheveled; PTCH, Patched; SMO, Smoothened; Sufu, Suppressor of Fused.

MODULATION OF WNT SIGNALING VIA GPC3

In HCC progression, activation of canonical Wnt signaling is a frequent molecular event (29). Approximately 95% of HCCs exhibit Wnt/β-catenin deregulation (30). The Wnt cascade is aberrantly activated in several human diseases including cancers and metabolic disorders. In humans, a total of 19 Wnts are secreted via autocrine and paracrine systems (31). Canonical Wnt signaling, a β-catenin-dependent process, is prompted by Wnt binding via two coreceptors: frizzled (FZD), a seven-pass transmembrane G protein coupled receptor (GPCR), and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), a single-pass transmembrane receptor. There are 10 total human FZDs (32, 33). Wnt ligands bind to FZD's protruding extracellular cystine-rich domain containing the Wnt binding domain. The interaction between Wnt and FZD promotes the assembly of the FZD-LRP5/6 receptor complex (34). Conformational changes in FZD and LRP5/6, followed by phosphorylation of glycogen synthase kinase 3 and casein kinase 1 promote recruitment of Axin, an important component of the destruction complex. Consequently, DVL, a cytoplasmic protein, is recruited and binds to the C terminal tail of FZD. Thus, destruction complex, containing DVL, Axin and other binding partners, is stabilized (35–38). Axin prevents β-catenin from degradation; therefore, β-catenin accumulates in the cytoplasm, travels to the nucleus, and drives transcription of cell proliferation and survival genes (**Figure 1**) (39). Wnt signaling can also act in a β-catenin independent fashion, termed the non-canonical or alternative pathway (40). Since Wnt signaling is vital for many functions such as hepatobiliary functions, cell differentiation, and repair, Wnt dysregulation can result in HCC, hepatoblastoma, cholangiocarcinoma, or other liver diseases.

In HCC cell lines, overexpression of GPC3 promotes the proliferation and growth, indicating that GPC3 regulates cell surface signaling by functioning as a co-receptor for Wnt proteins (26). Interestingly, both GPC3ΔGAG (the mutant GPC3 lacking the HS side chains) and GPC3 were able to form a complex, indicating that the GPC3-Wnt complex was enabled through the core GPC3 protein. GPC3 HS chains are not required for Wnt activation, but instead, the HS chains may be important to stabilize FZD (41). These data provided initial evidence of the interaction between GPC3 and Wnt. Our laboratory reported evidence of the potential mechanism of GPC3 enhancement of Wnt3a/β-catenin signaling activity in Hep3B and other HCC cells lines by blocking GPC3 by antibodies. HS20, a human monoclonal antibody isolated using

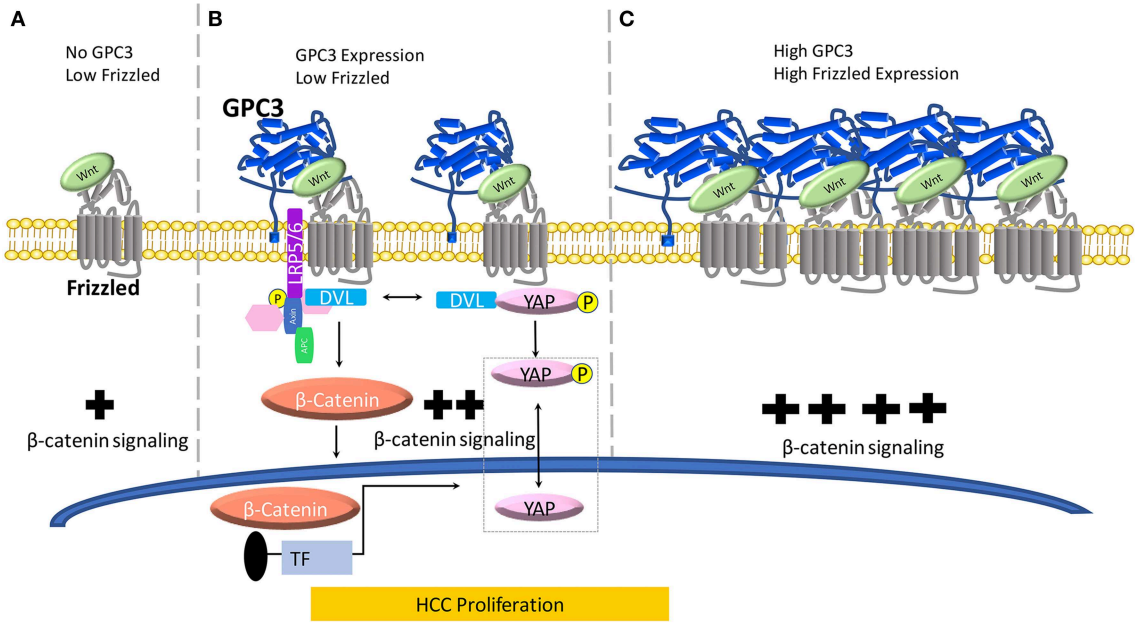

FIGURE 1 | A model for the role of GPC3 in regulating Wnt in liver cancer. **(A)** When no GPC3 is present, Wnt can independently activate FZD without GPC3 co-ordination. In the absence of GPC3, there is a baseline level of Wnt/β-catenin activation in normal liver cells. **(B)** When GPC3 is upregulated in malignant liver cells (HCC), GPC3 serves as a Wnt co-receptor to attract Wnt to the cell surface via a hydrophobic groove in the N-lobe of GPC3 containing F41 and surrounding residues. **(C)** When FZD is locally concentrated and GPC3 is high in HCC cells, the Wnt/GPC3/FZD complex is formed, and Wnt signaling is amplified.

phage display technology, blocks the Wnt3a/β-catenin cascade by binding the HS chains (10). The HS20 antibody interferes with binding of GPC3 to Wnt3a and impedes access to FZD. In the same study, we showed that HS20 inhibited Wnt/β-catenin signaling in HCC cell lines and cells which endogenously express GPC3. Then, using an *in vivo* model, our group showed that HS20 has considerable antitumor activity when nude mice were inoculated with Hep3B and HepG2 cells, separately (10). In another study, the endogenous interaction between GPC3 and Wnt was confirmed in the Hep3B model (42). Additionally, the oncogenic human sulfatase SULF2, which is upregulated in over 60% of HCCs and has, 6-O-desulfatase activity in mammalian cells, can release Wnt from HS chains and form a complex with GPC3 and Wnt. This provided an indication that sulfation of HS may play an essential role for binding Wnt and other growth factors. To understand the exact mechanism for the binding motif of Wnt on the HS glycans, we and collaborators devised an array of synthetic HS oligosaccharides with differing lengths and sulfation modifications (25). We found that 2-O and 6-O sulfations were essential for Wnt binding while 3-O sulfation could enhance Wnt binding, providing direct evidence for a Wnt binding domain on the HS chains on GPC3 (25). This work also provided mechanistic insights about the size of the Wnt binding domain which we estimated to be between 6 and 8 sugar residues. Taken together, these data reasonably link GPC3, SULF2, Wnt, and FZD. However, the precise Wnt binding domain on HS is yet to be shown by structural and functional studies.

Evidence of the Wnt binding domain on the GPC3 core protein has been suggested by using the HN3 single-domain antibody (27, 43). In a recent study, we and collaborators modeled Wnt/GPC3 to predict hydrophobic areas of interest (28). We identified, phenylalanine 41 (F41), a key residue within GPC3's hydrophobic groove located in the N-lobe of GPC3. We mutated the F41 residue as F41E and found it to be critical in recognizing Wnt3a in HCC cell and mouse models (28). Furthermore, in the same study, we showed that both major parts of GPC3, the core protein and HS glycan chains, can modulate Wnt signaling (**Figure 1**). In a Wnt functional reporter assay, overexpression of GPC3 alone activated Wnt signaling and could be lessened by the F41E mutation, but not by eliminating HS chains (**Figure 1B**). Interestingly, co-transfection of GPC3 and FZD induced synergistic activation of Wnt activity. This synergistic effect was stopped by removing the HS chains of GPC3, however the F41E mutation no longer showed any effect (28). This dynamic model can conceivably connect GPC3 expression and HCC progression in which low FZD and no GPC3 represents normal liver, high GPC3 and low FZD represents early stage HCC, and high FZD and high GPC3 coordination represents late stage HCC (44) (**Figure 1**). When GPC3 is upregulated in malignant liver cells (HCC), possibly by chronic inflammation due to hepatitis viral infection or other etiological factors (**Figure 1B**) (45), GPC3 serves as a Wnt co-receptor to attract Wnt to the cell surface via the newly identified cysteine-rich hydrophobic groove in the N-lobe of GPC3 containing F41. When FZD is locally concentrated and the Wnt/GPC3/FZD complex is formed, the HS component rather than the core protein of GPC3 can serve as a bridge for the stability of the complex (**Figure 1C**). In this way, GPC3 may act

as a bridge through its HS chains to stabilize Wnt and FZD after the Wnt/GPC3/FZD complex is formed. Thus, depending on the levels of FZD, GPC3 can promote Wnt activation through either the core protein or HS chains (**Figure 1**).

GPC3, WNT, AND YAP

Early work in the *Drosophila* model implicated the Hippo signaling pathway in modulating organ size and development. In mammals, the Hippo cascade involves two main kinases Mst1/2 and Lats1/2. Once these kinases are in play, Lats1/2, phosphorylates, YAP, a transcriptional co-activator. YAP inactivation leads to downregulation of target genes including: cyclin E, diap1, and bantam. YAP has been shown to be a critical nuclear effector within Hippo signaling, however, the precise mechanism by which Hippo signaling inactivates YAP function in mammals remains unclear (46). Furthermore, recent advancements in understanding signaling pathways have indicated that the Hippo pathway suppresses liver overgrowth and HCC development. YAP function is critical in regulating cell size, tissue regeneration, and cancer morphogenesis. Studies have indicated a connection between GPC3 and Wnt via YAP however the mechanism of crosstalk between β-catenin and YAP remains undetermined (**Figure 1B**). Additionally, in the cytoplasm, there is evidence that YAP can regulate DVL (47). Moreover, in liver cancer, HCC tissues showed higher YAP activation, indicating a positive correlation between HCC progression and YAP activity (48).

Our laboratory used phage display technology to identify the human single domain antibody, HN3, a GPC3 target, and showed that HN3 potently inhibited HCC cell growth. When investigating the mechanism of HN3 activity, we found that phosphorylated YAP (p-YAP), the inactive version of YAP, was greater in HCC cells treated with HN3. Overall, total YAP level was reduced in HCC cells treated with HN3. GPC3 knockdown led to lower cell proliferation and reintroduction of recombinant YAP was able to rescue the cells from apoptosis triggered by GPC3 knockdown (49). The observation is consistent with our early finding that GPC3 regulated YAP signaling in HCC cells (43). When we knocked down YAP in HCC cells, cell proliferation decreased by ∼50%. Upon subsequent HN3 treatment, YAP-knockdown cells did not further inhibit cell proliferation, indicating that YAP knockdown may cause acquired resistance to HN3 treatment. However, in mutant YAP overexpression cell lines where YAP is constitutively active, we reported increased cell proliferation and abatement of HN3 antagonist activity, i.e., HN3 could not inhibit cell proliferation. In a subsequent work, used a reporter assay to investigate YAP activity. In Hep3B cells, blocking Wnt via HN3-GPC3 binding also blocks YAP, overall indicating that Wnt may be involved in the upstream regulation of YAP signaling (27). Taken together, these results indicate that YAP is not only involved in HCC proliferation but also that GPC3 may act as an upstream regulator of YAP. As discussed previously, Li et al. reported that the HN3 antibody recognizes the Wnt binding site, a unique conformational epitope which is a cysteine-rich, hydrophobic groove in the N-lobe of GPC3 (28, 43). These works reasonably link YAP inactivation to GPC3 and Wnt via HN3.

GPC3, WNT, AND HEDGEHOG SIGNALING

The hedgehog (Hh) signaling pathway (**Figure 2**) plays a defining role in embryonic development and is highly conserved across species. Hh signaling is involved in cell growth, differentiation, tissue patterning, and vascularization. When aberrantly activated, these processes can lead to tumor growth, malignant transformation or metastasis. Thus, hyperactivation of the Hh pathway has been linked to various cancers including breast, prostate, liver, pancreatic, and brain (50, 51). In exploring the biological role of GPC3 in liver cancer (HCC) cells, GPC3 was found to promote HepG2 cell proliferation through Hh signaling (52).

The Hh signaling pathway involves recruitment of Hh ligands including: desert hedgehog (Dhh), Indian hedgehog (Ihh), and Sonic hedgehog (Shh). Any Hh ligand can initiate binding to the 12 transmembrane proteins Patched (PTCH) and various co-receptors, thus triggering Hh signaling by Smoothened(SMO) de-repression. SMO, a 7-pass transmembrane protein from the FZD family of GPCRs, mediates downstream signaling. Sufu, a cytoplasmic protein, and GLI proteins, main transcriptional effectors, cooperate to induce Hh activation and expression of Hh target genes. In the absence of Hh ligands, or in the case of Hh ligands binding to GPC3, PTCH will not be active and SMO will be repressed, thereby inhibiting Hh signaling (**Figure 1B**) (53, 54).

Early work in the *Drosophila* model demonstrated that glypicans are involved in regulation of Hh signaling (53). GPC3-null mice, a SGBS disease model, display increased Hh signaling activity and higher levels of Shh and Ihh (55). Further, GPC3 binds to Shh and Ihh with high affinity and competes with PTCH for Hh binding (55). Of note, the core protein of GPC3 can directly bind Hh to inhibit its signaling activity in cell culture (26). In a later study, experimental evidence demonstrated that cleavage by convertases is also crucial for GPC3 inhibition of Hh signaling (56). Low-density-lipoprotein receptor-related protein-1 (LRP1) was also shown to mediate endocytosis of the GPC3-Hh complex (57) (**Figure 2B**).

CD81, a cell surface tetraspanin, which facilitates Hepatitis C Virus (HCV) entry into hepatocytes, further entangles GPC3 with Hh and Hippo signaling. CD81 is main GPC3 binding partner and the GPC3/CD81 interaction modulates Hh signaling through hematopoietically expressed homeobox protein (Hhex), a transcriptional repressor (58, 59). However, 78% of HCCs do not express CD81 indicating loss of CD81 expression occurs commonly in HCCs (60). In the JM2 rat hepatoma cell line, forced expression of CD81 in the presence of high GPC3 expression, increased activation of the Hippo pathway by decreasing nuclear YAP (60). The precise connections between these signaling pathways remain unclear and future work which elucidate the interplay between YAP, Wnt, hedgehog, and other signaling players will be necessary in designing targeted therapeutics. Since the outlined signaling pathways all have

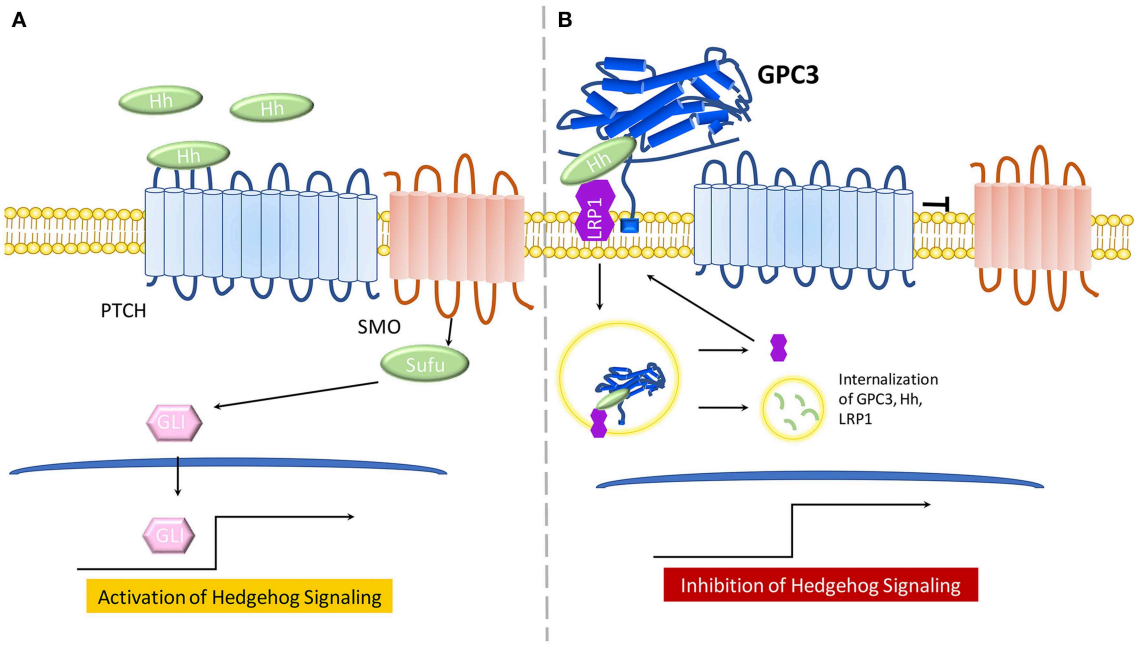

FIGURE 2 | The model for the role of GPC3 in regulating Hedgehog signaling. **(A)** In the presence of Hh ligand, PTCH promotes surface localization and activation of SMO. SMO transduces the Hh signal within the cytoplasm. Protein kinases phosphorylate GLI proteins, leading to an NH_2-terminal truncated form, which travels to the nucleus and activates Hh signaling. **(B)** GPC3 competes with PTCH for Hh binding which results in inhibition of hedgehog signaling. GPC3 binds both Shh and Ihh, resulting in the endocytosis and lysosomal degradation of the GPC3/Hh complex in the presence of LRP1.

underlying roles in cell growth and proliferation processes, cross regulation is an essential strategy. Therefore, key steps in Wnt, YAP and hedgehog may be connected via GPC3 and its counterparts to tightly control fundamental cellular processes as a fine-tuning mechanism (50, 54, 61, 62).

FUTURE PERSPECTIVES

GPC3 is clearly an important player Wnt, Hh, and YAP signaling cascades. However, fundamental questions regarding the GPC3/Wnt/FZD complex structure, intratumor heterogeneity of protein expression, and alternatively spliced variants of GPC3 in liver cancer have yet to be fully understood. Future work addressing the mechanism of GPC3 in the outlined signaling pathways would provide a more complete picture of its precise role in oncogenesis of liver cancer. Rigorous experimental interrogation of mechanism will be crucial in engineering therapies which can disrupt tumor progression. Nevertheless, as extensively summarized in other recent articles, the development of GPC3-targeted therapies has emerged with many clinical trials worldwide (15, 16, 63). These ongoing clinical trials will help define the utility of GPC3 as a target for liver cancer therapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The National Cancer Institute (NCI) holds patent rights to anti-GPC3 antibodies including HN3, YP7, and HS20 in many jurisdictions, including the USA (e.g., US Patent 9409994, US Patent 9206257, US Patent 9304364, US Patent 9932406, US Patent Application 62/716169, US Patent Application 62/369861), China, Japan, South Korea, Singapore, and Europe. Claims cover the antibodies themselves as well as conjugates that utilize the antibodies, such as recombinant immunotoxins (RITs), antibody–drug conjugates (ADCs), bispecific antibodies, and modified T cell receptors (TCRs)/chimeric antigen receptors (CARs) and vectors expressing these constructs. Anyone interested in licensing these antibodies can contact MH (NCI) for additional information.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Unraveling Heparan Sulfate Proteoglycan Binding Motif for Cancer Cell Selectivity

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Membrane heparan sulfate proteoglycans (HSPG) regulate cell proliferation, migration, and differentiation and are therefore considered key players in cancer cell development processes. Here, we used the NT4 peptide to investigate how the sulfation pattern of HSPG on cells drives binding specificity. NT4 is a branched peptide that binds the glycosaminoglycan (GAG) chains of HSPG. It has already been shown to inhibit growth factor-induced migration and invasiveness of cancer cells, implying antagonist binding of HSPG. The binding affinity of NT4 with recombinant HSPG showed that NT4 bound glypican-3 and -4 and, with lower affinity, syndecan-4. NT4 binding to the cancer cell membrane was inversely correlated with sulfatase expression. NT4 binding was higher in cell lines with lower expression of SULF-1 and SULF-2, which confirms the determinant role of sulfate groups for recognition by NT4. Using 8-mer and 9-mer heparan sulfate (HS) oligosaccharides with analog disaccharide composition and different sulfation sites, a possible recognition motif was identified that includes repeated 6-O-sulfates alternating with N- and/or 2-O-sulfates. Molecular modeling provided a fully descriptive picture of binding architecture, showing that sulfate groups on opposite sides of the oligosaccharide can interact with positive residues on two peptide sequences of the branched structure, thus favoring multivalent binding and explaining the high affinity and selectivity of NT4 for highly sulfated GAGs. NT4 and possibly newly selected branched peptides will be essential probes for reconstructing and unraveling binding sites for cancer-involved ligands on GAGs and will pave the way for new cancer detection and treatment options.

Keywords: heparan sulfate proteoglycans, peptide, tumor targeting, sulfatase, oligosaccharide

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INTRODUCTION

Heparan sulfate proteoglycans (HSPG) are a large family of heterogeneous molecules found in the extracellular matrix (ECM) and on the membranes of vertebrate cells. They are composed of a protein linked to sulfated glycosaminoglycan (GAG) chains, which are linear polymers of repeated disaccharide units consisting of an amino sugar and uronic acid, that can be modified with sulfate groups at various positions. HSPG can be classified by their localization as extracellular, intracellular, pericellular, and cell surface associated. Cell surface HSPG include the two families of syndecans and glypicans and betaglycan, a transmembrane proteoglycan (PG) with heparan

and chondroitin sulfate chains. Glycosaminoglycan moieties in membrane-associated HSPG do not differ much in saccharide composition but are very different in sulfation pattern in terms of positions and number of sulfates (1, 2). Since membrane HSPG regulate cell proliferation, adhesion, migration, and differentiation (3, 4), they are considered key players in cancer cell development (1). This is because GAG chains of HSPG interact with a large number (>435) of extracellular regulatory proteins, such as growth factors, chemokines, and morphogens (5). Indeed, drugs directed against HSPG are being evaluated in preclinical models. For example, peptides directed against syndecan-1 have shown therapeutic promise in preclinical models of breast cancer and myeloma (6–8).

NT4 peptide is a tetrabranched peptide that binds to GAG chains of HSPG. Its branched structure, obtained by synthesizing four copies of the 13-amino-acid sequence on a branching core of lysines, makes NT4 stable to proteolytic enzymes and gives it a long half-life (9, 10). NT4 binds cell lines of different human cancers, including colon adenocarcinoma, pancreas adenocarcinoma, bladder cancer, and breast cancer (11, 12). It does not bind PgsA-745 cells (Chinese hamster ovary cell mutant), which lack GAG chains, being deficient in xylosyltransferase, the enzyme responsible for anchorage of GAG chains to the protein core (13). Tumor selectivity was very evident in surgical resections of colon, pancreas, and bladder cancer, stained with NT4 conjugated with a fluorescent probe, compared to the healthy counterparts (14–16).

NT4 peptides can be conjugated with different functional units and can selectively deliver drugs for cancer therapy or transport tracers for tumor imaging (11, 12, 15–18). Using drug-conjugated NT4, we obtained a significant reduction in tumor growth or even tumor regression (11, 14, 17), compared to animals treated with the unconjugated drug under identical conditions. NT4 transports the chemotherapeutic moiety to the cancer cell membrane and, ultimately, into the cell (14–16). In animal models of cancer, the higher concentration of the cytotoxic drug at the site of the tumor, obtained by the targeting with the peptide, showed better efficacy than the free drug (11, 14, 17). We found that the high selectivity of NT4 toward cancer cells and tissues resides in its high-affinity binding to sulfated GAGs, with preferential high-affinity binding to heparin and heparan sulfate (HS) compared to chondroitin sulfate (CS) (13, 19). Importantly, NT4 inhibited oriented migration of pancreas adenocarcinoma cells (13) as well as growth factor-induced migration and invasiveness of breast cancer cells, implying antagonist binding to HSPG (13, 20).

Here, we report how the sulfation pattern of HSPG on cells can drive binding specificity. Regardless the expression of different HSPG on cancer cells, GAG linear polymers are the only exposed HSPG moiety on the outer membrane and are responsible for specificity.

The glycoside sequence and sulfation pattern of GAGs are crucial for ligand binding and are synthesized by enzymes in the Golgi apparatus and modified by extracellular enzymes that can introduce recognition patterns for growth factors (2) and other binding proteins. The specificity of GAG–ligand interactions has been reported in several studies. For example, it has been described in the case of the fibroblast growth factor (FGF)–heparin interaction, where the key residues on FGF and GAG chains were identified (21). The FGF–HS–FGFR1 ternary complex can only be formed in the presence of 6-O-sulfate groups on HS (22, 23). Interestingly, it has been observed that short analogs of heparin, i.e., heparin oligosaccharides, featuring one or two 6-O-sulfate groups on the reducing end of glucosamine, can fully activate FGF2 signaling (24). 6-O-sulfation of HS is also reported to be necessary to prompt the response of primary fibroblasts to transforming growth factor-β1 (TGFβ1), whereas 6-O-sulfates negatively regulate Wnt signaling (25, 26).

NT4 binds a specific pattern and competes with GAG binding proteins for important biological functions like angiogenesis and migration. As such, NT4 was used here to define the fine structure of binding sites on GAG chains.

METHODS

Peptide Synthesis

Peptides were synthesized on an automated multiple synthesizer (MultiSynTech, Germany) by standard Fmoc chemistry. NT4 was synthesized on $Fmoc_4$-Lys_2-Lys-beta-Ala-Tentagel resin (Rapp Polymer) using protected L-amino acids (Iris Biotech), DIPEA (N,N-diisopropylethylamine) (Merck), and HBTU (hexafluorophosphate benzotriazole-N,N,N',N'-tetramethyl uronium) (MultiSynTech). Pyro-Glu-O-pentachlorophenylester (Bachem, Switzerland) was used for the last coupling step. NT4-biotin was synthesized on Tentagel resin with Fmoc-Lys(biotin)-OH as the first coupling step, and Fmoc-PEG_{12}-OH as the second; Fmoc-Lys(Fmoc)-OH was then used to build the tetrameric core. At the end of the coupling sequence, peptides were cleaved from the resin, deprotected, and lyophilized.

High-performance liquid chromatography (HPLC) purification was performed on a C18 Jupiter column (Phenomenex). Water with 0.1% trifluoroacetic acid (TFA) (A) and methanol (B) were used as eluents. Linear gradients over 30 min were run at flow rates of 0.8 and 4 ml/min for analytical and preparatory procedures, respectively. All compounds were also characterized on a BrukerUltraflex matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass spectrometer.

NT4 $(pyELYENKPRRPYIL)_4K_2K$-beta-Ala MS: *m/z* calculated for $C_{333}H_{519}N_{91}O_{81}$ $[M+H]^+$ was 7,094.24; detected 7,095.15. HPLC RT (from 80 to 20%A) 26.63 min. NT4-biotin $(pyELYENKPRRPYIL)_4K_2K$-PEG_{12}-K(biotin) MS: *m/z* calculated for $C_{373}H_{594}N_{96}O_{95}S$ $[M+H]^+$ was 7,976.35; detected 7,978.72. HPLC RT (from 80 to 20%A) was 26.99 min.

Cell Lines

PANC-1 human pancreas adenocarcinoma, HT-29 human colon adenocarcinoma, and MCF-7 and MDA-MB-231 human breast adenocarcinoma cells were grown in the recommended American Type Culture Collection (ATCC) media, supplemented with 10% fetal calf serum, 200 μg/ml glutamine, 100 μg/ml streptomycin, 60 μg/ml penicillin, and maintained at 37°C, 5% CO_2. Cell lines were purchased from

ATCC, and cell profiling was analyzed to authenticate human cell lines (BMR Genomics).

Flow Cytometry

All experiments were performed using 2×10^5 cells in 96-well U-bottom plates. All dilutions were performed in phosphate-buffered saline (PBS), containing 5 mM EDTA and 1% bovine serum albumin (BSA).

NT4 Binding

Cells were incubated with 1 μM NT4-biotin for 30 min at room temperature and then incubated with 1 μg/ml streptavidin–fluorescein isothiocyanate (FITC). For heparinase treatment, cells were incubated for 1 h at 37°C on the plates with 0.03 IU/ml heparinase I/III blend (Sigma Aldrich), and then harvested and incubated with the same concentration of heparinase in suspension for an additional hour at 37°C before NT4 staining. All experiments were repeated two times. *P* values were calculated using a two-tailed Student *t*-test and GraphPad Prism 5.0 software.

Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA samples were extracted from different human cancer cells (1×10^6 cells) with TRIzol (Invitrogen, Milan, Italy). For quantitative RT-PCR, RNA samples were retrotranscribed using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, Monza, Italy) and amplified on an Abi Prism 7000 instrument (Applied Biosystems, Monza, Italy) using the TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions.

The following human TaqMan gene expression assays were used: glypican-3 GPC3 (Hs00170471_ml), glypican-4 GPC4 (Hs00155059_m1), syndecan-3 SDC3 (Hs00206320_m1), syndecan-4 SDC4 (Hs00161617_m1), and β-actin (Hs99999903_m1). Fluorescent signals generated during PCR amplifications were monitored and analyzed with the Abi Prism 7000 SDS software (Applied Biosystems). The following PCR conditions were applied: 50°C for 2 min, 95°C for 10 min, and 40 amplification cycles (95°C for 15 s and 60°C for 60 s).

In order to determine the efficiency of each TaqMan gene expression assay, standard curves were generated by serial dilution of cDNA, and quantitative evaluations of target and housekeeping gene levels were obtained by measuring threshold cycle numbers (Ct). A relative quantitative analysis was performed, using the $2^{-\Delta\Delta Ct}$ value, where ΔCt = Ct (target)—Ct (endogenous control) and $\Delta\Delta Ct$ = ΔCt (sample)—ΔCt (calibrator). Beta actin was used as an endogenous control, and the sample with the lowest expression was used as a calibrator (syndecan-3 in HT-29).

Gene Expression of Human Sulfatases by RT-PCR

PANC-1, HT-29, MDA-MB-231, and MCF-7 cells were seeded in 6-well plates (5×10^5 cells per well) and cultured overnight in a CO_2 incubator. Total RNA was extracted using an RNA isolation kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was quantified by spectrophotometry at 260 and 280 nm and verified by agarose gel electrophoresis. The same quantity of RNA for every cell line was loaded on the gel. One-step RT-PCR (QIAGEN) was applied for retrotranscription and human cDNA amplification of SULF-1 (393 pb) and SULF-2 (434 pb). The following oligonucleotides were used as primers: SULF-1 primers, 5'-ACTTCCACTGCCTGCGTAATGA-3′ (sense) and 5′-ATGAACGCTTTGAGGCTAGGCA-3′ (antisense); SULF-2 primers, 5′-CCCAGAAGCTCACAAAGGAAAACG-3′ (sense) and 5′-AATGTCCACAACTGCGAGGGAT-3′ (antisense).

The following PCR conditions were applied: for SULF-1, 30 denaturing cycles at 94°C for 60 s, annealing at 58°C for 60 s, and extension at 72°C for 90 s; for SULF-2, 30 denaturing cycles at 94°C for 60 s, annealing at 54°C for 60 s, and extension at 72°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as experimental control. Signals were detected using Image LAS4010 (GE Healthcare). Densitometry analysis was carried out using ImageJ software. The value 100% corresponds to GAPDH gene expression for each cell line. The experiment was performed twice. *P* values were calculated using a one-tailed Student *t*-test and GraphPad Prism 5.0 software.

Expression of Sulf-1

HT-29, PANC-1, MDA-MB-231, and MCF-7 cells were seeded in 6-well plates (1.5×10^6 cells per well), previously coated with 10 μg/ml plasma fibronectin, and maintained overnight in a CO_2 incubator. Cells were lysed according to the antibody supplier's instructions (Abcam). Total proteins (20 μl/lane) were separated with a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was saturated with 5% w/v nonfat dry milk in PBS containing 0.1% Tween20 for 1 h at room temperature and then incubated with specific antibodies [rabbit polyclonal to sulfatase 1/SULF-1 antibody (1 μg/ml, Abcam), and mouse anti-GAPDH monoclonal antibody (1 μg/ml, Invitrogen)]. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000, Cell Signaling) in the case of anti-sulfatase 1/SULF-1 antibody and with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:10,000, ThermoFisher). Signals were detected using Image LAS4010 (GE Healthcare). Densitometry analysis was carried out using ImageJ software. The value 100% corresponds to average GAPDH protein expression for the four cell lines. The experiment was performed three times. *P* values were calculated using a parametric, unpaired Student *t*-test, and GraphPad Prism 5.0 software.

Surface Plasmon Resonance (SPR) Experiments

Experiments were performed on a Biacore T100 instrument (GE Healthcare). All materials were purchased from GE Healthcare unless otherwise specified. Full-length recombinant human HSPG were purchased from R&D Systems. Syndecan-3, syndecan-4, and glypican-3 were obtained from the mouse myeloma cell line (NS0), and glypican-4 was obtained from the

Chinese Hamster Ovary cell line. The activity of syndecan-4, glypican-3, and glypican-4 was measured by the supplier as the ability of the immobilized protein to bind FGF-basic. The activity of syndecan-3 was measured by the supplier as the ability of the immobilized protein to inhibit adhesion of Saos-2 human osteosarcoma cells to human fibronectin.

Eight-mer and nine-mer oligosaccharides S00 $(GlcNAc\text{-}GlcA)_4$ α-paranitrophenyl, S04 $(GlcNS\text{-}GlcA)_4$ α-paranitrophenyl, S06a $(GlcA\text{-}GlcNS)_2\text{-}(GlcA\text{-}GlcNS,6S)_2\text{-}GlcA$ α-paranitrophenyl (9-mer), and S06b GlcNS-GlcA-GlcNS-IdoA,2S-GlcNS-IdoA,2S-GlcNS-GlcA α-paranitrophenyl were purchased from Iduron. In all oligosaccharides, the units were linked together by α (1–4) bonds only and carry a paranitrophenyl group. S12 $(\Delta HexA,2S\ \alpha 1\text{-}4\ GlcNS,6S)_3$ (9-mer) was from Amsbio, and its first glycoside is unsaturated.

NT4-biotin was captured on a CM5 sensor chip where streptavidin had previously been immobilized by standard amine coupling. Briefly, the sensor chip surface was activated with a mixture of 0.1 M 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.4 M N-hydroxyl succinimide (NHS) for 7 min at a flow rate of 5 µl/min. Streptavidin was injected over the surface for 7 min, and finally, 1 M ethanolamine pH 8.5 was used to block any activated carboxyl groups. NT4-biotin, diluted in HBS-EP+ (Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM, 0.05% p20, pH 7.4) to 30 µg/ml, was injected for 2 min at a flow rate of 10 µl/min.

HSPG and oligosaccharides were diluted to different concentrations in HBS-EP+ and then injected over immobilized NT4 peptides. The sensor chip surface was regenerated with a short pulse of 10 mM NaOH/0.5 M NaCl 5 min after the end of the injections.

Kinetics were analyzed with the Biacore T100 evaluation 1.1.1 software using the 1:1 Langmuir model to fit the curves.

Modeling of NT4-Sulfated Oligosaccharide Complex

NT4 was modeled as extended conformation structure using PyMOL (The PyMOL Molecular Graphics System, Version 1.4, Schrödinger, LLC) and refined by energy minimization with the Gromacs package (27) and Amber force field (28). The molecule was centered in a triclinic box with at least 10-Å distance from the solute to the periodic box border; the box was filled with TIP3P water model, and the system was neutralized by adding counterions. A new force field entry was created for lysine in the scaffold by reparameterization of the standard lysine residue from the Amber library, taking covalent bonding of the side-chain amine into account. The peptide was linked to available amines of the scaffold. The three-dimensional (3D) structure of the 8-mer heparin oligosaccharide was derived from the canonical helical structure of heparin (PDB ID 1HPN, $^1C_4$ conformer) (29). The GLYCAM06 force field parameters (30) were used for GAGs.

RESULTS

In previous papers, we reported NT4 binding and internalization into different cancer cell lines by immunofluorescence and flow cytometry (11, 13, 14, 19). In previous confocal microscopy experiments, NT4 conjugated with biotin (NT4-biotin) already proved to be completely internalized only after 2 h at 37°C (14, 16). Degradation of NT4-biotin by living cells was previously assessed by mass spectrometry and showed that the molecule was still stable after 24 h (14). NT4 binding and internalization into those cancer cells or tissues were completely inhibited by heparin and HS (13, 19). We also demonstrated that NT4 binds to heparin and HS with high affinity and to CS with lower affinity (13).

To further assess the specificity of binding of the NT4 peptide to HSPG in HT-29 colon adenocarcinoma, PANC-1 pancreas adenocarcinoma, and MDA-MB-231 and MCF-7 breast cancer human cell lines, we first treated the cells with the heparinase I/III blend that removes HS from proteoglycans. We then incubated the cells with NT4. Flow cytometry analysis showed that NT4 binding to cancer cells treated with heparinase was much lower than to control cells (**Figure 1**).

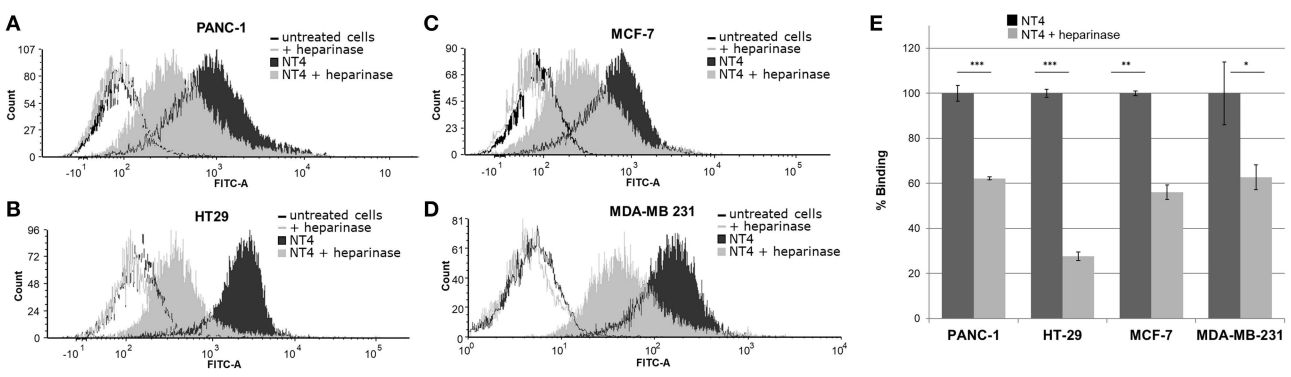

FIGURE 1 | (A–D) NT4 binding to PANC-1, HT-29, MCF-7, and MDA-MB-231 cancer cells before and after heparinase I/III treatment, tested by flow cytometry. **(E)** Variation of binding of NT4 after heparinase treatment, 100% (dark histogram) is the baseline binding of NT4 to the different cell lines $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ by Student's *t*-test.

Gene Expression of Glypicans and Syndecans in HT-29, PANC-1, MDA-MB-231, and MCF-7 Cancer Cells

Glypican and syndecan levels have recently been studied with a view to defining new tumor markers or prognostic tools (6, 31). Elevated levels of glypican-1 are found in pancreas carcinoma where increased expression is associated with poor prognosis (32). Levels of glypican-1 and syndecan-2 are also increased in colorectal cancer (1). Breast cancer was found to upregulate glypican-1 (33–35) and syndecan-4 (36) and to downregulate glypican-3 (37), while loss of glypican-3 promotes tumor proliferation and metastasis (38). Glypican-2 is upregulated in neuroblastoma and associated with poor overall survival (1). The roles of glypican-4 and syndecan-3 in tumors are still underexplored.

Figure 2 shows syndecans and glypicans expression in HT-29, PANC-1, MDA-MB-231, and MCF-7 as analyzed by qRT-PCR.

Expression of syndecans (**Figure 2**, shades of green) was generally higher than that of glypicans (**Figure 2**, shades of blue). Among syndecans, syndecan-4 was the most expressed in all cell lines, followed by syndecan-3 in MCF-7, MDA-MB-231, and PANC-1 cells. Among glypicans, glypican-4 was the most expressed, but only in MCF-7 cells (**Figure 2**).

Sulfatases Modulate NT4 Binding on Cancer Cells

Human sulfatase 1 (hSULF-1) and human sulfatase 2 (hSULF-2) are extracellular enzymes that remove 6-O-sulfate groups from HS chains. Modified expression of both sulfatases, particularly SULF-1, has been associated with different cancers (38). By hydrolyzing 6-O-sulfate groups, hSULF-1 and hSULF-2 modulate binding of HS-binding proteins, such as growth factors and cytokines, and, finally, have effects on cell signaling (38). For example, hSULF-1, acting on HS, reduces the formation of the FGF2–FGFR–HS complex and consequently impairs FGF2 signaling (39).

Figure 3A shows the relative abundance of mRNA of hSULF-1 and hSULF-2 in HT-29, PANC-1, MCF-7, and MDA-MB-231 cells as measured by RT-PCR. The two sulfatases were expressed very differently in the different cell lines. SULF-1 protein expression was also measured in the same cell lines using a specific anti-SULF-1 antibody (**Figure 3B**). PANC-1 and HT-29 cells showed much lower expression of sulfatases, which implies that their sulfated GAG chains retain more 6-O-sulfate groups than cancer cells with higher expression of sulfatases, such as MCF-7 and MDA-MB-231.

The pattern of NT4 cell binding detected by flow cytometry (**Figure 3C**) suggests that cells expressing lower levels of sulfatases, particularly SULF-1, such as PANC-1 and HT-29, bind NT4 better than the others. The higher presence of the 6-O-sulfate groups is therefore correlated with higher binding of NT4 to those cell lines.

Affinity of NT4 for Recombinant HSPG and Sulfated GAGs

We used SPR to measure the affinity of NT4 binding to recombinant syndecans and glypicans, selected among those highly expressed by HT-29, PANC-1, MDA-MB-231, and MCF-7 cancer cell lines. We found that NT4 does not bind syndecan-3, whereas it binds syndecan-4, glypican-3, and glypican-4 (**Figures 4A–D**) with different affinities, the affinity of both glypicans being five times greater than that of syndecan-4. SPR analysis also enabled kinetic evaluation of NT4 binding to HSPG, showing different kinetic rates of association and dissociation (**Table 1**).

Binding of NT4 to synthetic oligosaccharides carrying different sulfation patterns was also analyzed. We used 8-mer and 9-mer oligosaccharides with different sulfation patterns: no sulfation in oligosaccharide S00, 4 N-sulfate groups in S04, 6 sulfate groups in S06a including 4 N-sulfates and 2 6-sulfates, 6

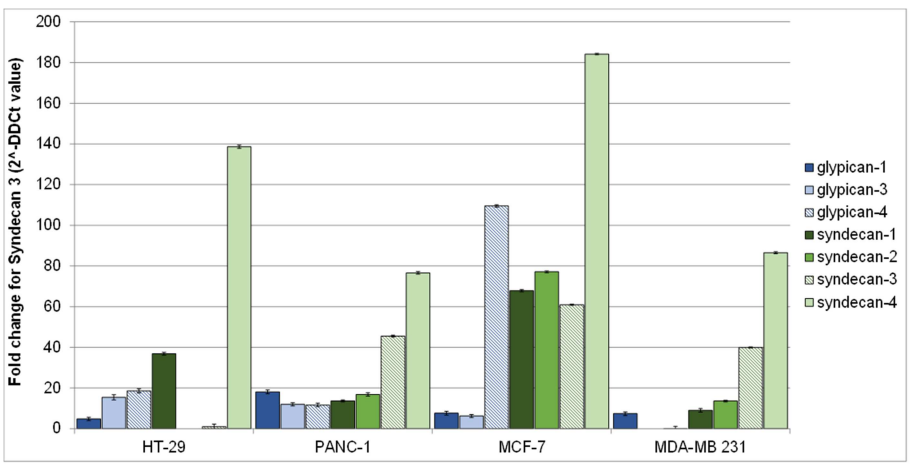

FIGURE 2 | Gene expression of human glypicans (shades of blue) and syndecans (shades of green) in HT-29, PANC-1, MCF-7, and MDA-MB-231 human cancer cell lines determined by qRT-PCR and normalized against β-actin. Results are reported as fold change for syndecan-3 in HT-29.

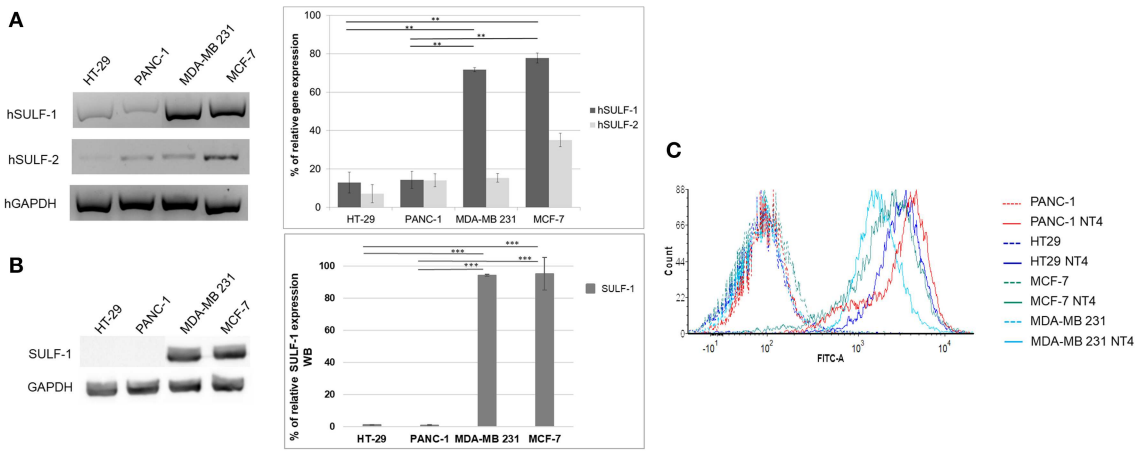

FIGURE 3 | (A) Gene expression of hSULF-1 and hSULF-2 in HT-29, PANC-1, MCF-7, and MDA-MB-231 cell lines analyzed by RT-PCR. The GAPDH gene was tested as endogenous control. Histograms represent percentage of gene expression compared to GAPDH (GAPDH is 100%). **(B)** SULF-1 expression analyzed by Western blot in the same cell lines. Histograms represent percentage of SULF-1 expression compared to GAPDH (GAPDH is 100%). Significance of the differences was calculated using Student's *t*-test and GraphPad, $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** NT4 binding analyzed by flow cytometry in HT-29, PANC-1, MCF-7, and MDA-MB-231.

sulfate groups in S06b including 4 N-sulfates and 2 2-O-sulfates, and, finally, 12 sulfate groups in S12, 4 in 6-O-position, 4 in 2-O, and 4 in N. We observed that the more sulfate groups there were, the higher was the affinity of the oligosaccharide for the peptide. We also found a correlation between sulfation in position 6 of oligosaccharides and NT4 binding affinity. Indeed, S06a, which carries the same number of sulfates as S06b, bound NT4 better by virtue of having two 6-O-sulfates (**Figure 4**). The best-binding oligosaccharide was S12, which carries repeated 6-O-sulfates, like S06a, but the 6-O-sulfates in S12 are alternated with 2-O or N-sulfates, making binding more stable (**Table 1**).

Graphical Model of Interaction of NT4 and a Sulfated Oligosaccharide

NT4 was modeled with PyMol and refined by energy minimization. The 3D structure of the positively charged stretch of the NT4 peptide sequence (K6PRRP10), previously demonstrated to be critical for heparin binding (19), resulted in an extended conformation that lowers steric hindrance between rigid prolines and their preceding amino acids bearing a large side chain. This conformation gives rise to a triangular pattern formed by the charged termini of K6, R8, and R9, with 6–8 and 8–9 distances of ∼12 Å and an angle of ∼130° between residues 6–8–9.

The 8-mer oligosaccharide was chosen for the *in silico* study on the basis of the experimental result obtained with flow cytometry that identified S12 (12 sulfate groups in an 8-mer) as the best-binding oligosaccharide, and its 3D structure was derived from the canonical helical structure of heparin (PDB ID 1HPN, $^{1}C_4$ conformer) (29).

Previous studies showed that the binding of heparin and HS to polypeptides is ionic in nature (40–42). The charge-based interactions between the acidic substituents on the polysaccharide and basic residues on the polypeptide are reported to dominate the interface, and charges have to be in an appropriate 3D pattern (43). For example, FGF1 proved to prefer a specific pattern of sulfate groups in a specific spatial distribution (44). Following such evidences, a matching between charge clusters was attempted by mean of 3D molecular graphics.

Indeed, the sulfates of $GlcNS_{i-3}$-IdoA2S$_i$-GlcNS6S$_{i+1}$ (corresponding to $GlcN_2$-IdoA$_5$-GlcN$_6$ and $GlcN_4$-IdoA$_7$-GlcN$_8$), lying on the same side of the helix, form a pattern with distances and angles coherent with those of charged side chains of KPRR, and a specific geometry of interaction of charges is suggested (yellow dashed lines in **Figure 5**). Similar results hold for the $^{1}C_4$ and $^{2}S_0$ cyclic forms of the oligosaccharide. On an 8-mer saccharide, this pattern is found twice on opposite sides of the helix, possibly interacting with two different NT4 peptide arms.

This interaction model also explains the almost total loss of binding for S04 (N-sulfates only), where alternate side sulfates are unable to form any negative charge cluster (**Figure 5**) that could fit with the positive cluster of NT4.

The *in silico* modeling provides a theoretical picture of the interaction that can help in understanding the binding activity of NT4. In particular, the fact that the oligosaccharide has two negative clusters on opposite sides of the molecule could reinforce the hypothesis of multiple binding with NT4.

DISCUSSION

HSPG are synthesized by most animal cells, but due to the variable composition and sulfation of their GAG chains, their ability to interact with specific ligands may be modulated under different physiological and pathological conditions, including cancer. Tumor stroma is composed of the ECM,

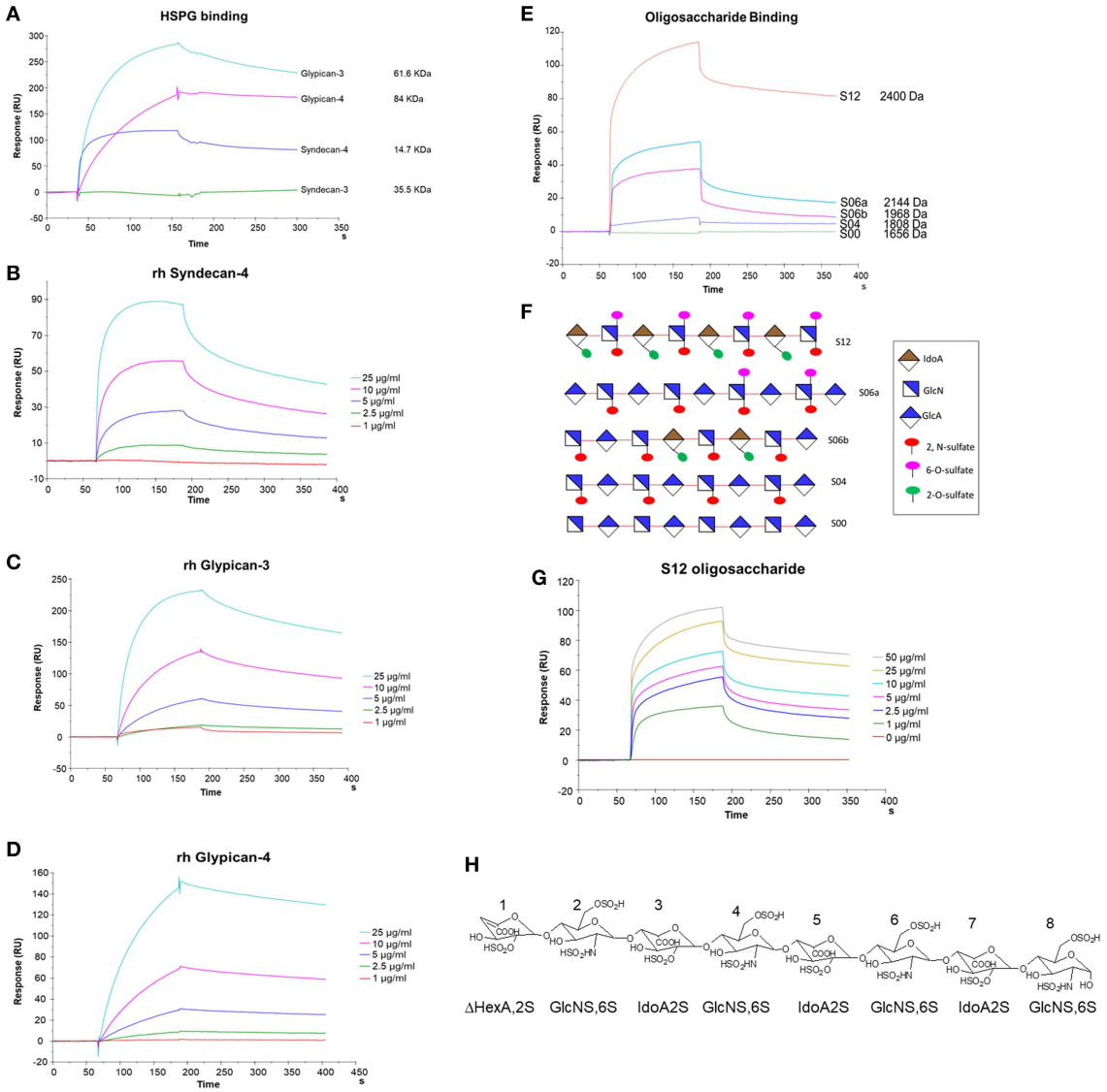

FIGURE 4 | SPR analysis of rHSPG and oligosaccharide binding to NT4. **(A)** rHSPG binding (25 µg/mL) to immobilized NT4. **(B–D)** Affinity of rHSPG for NT4. **(E)** Oligosaccharide (100 µg/ml) binding to surface immobilized NT4. **(F)** Schematic representation of oligosaccharides with sulfation sites. **(G)** Affinity of S12 sulfated oligosaccharide binding to NT4. **(H)** Structure of S12.

including proteoglycans, fibronectin, collagen, cytokines, and growth factors. Cells that populate the tumor stroma, like immune system cells, fibroblasts, and endothelial cells, together with tumor cells, can modify the stroma as the tumor evolves. The ECM of the tumor stroma is very different from that of normal tissues (1) due to tumor remodeling that also triggers tumor invasiveness (1). HSPG accumulate in remodeled stroma and are, in turn, modified on their glycosidic chains by tumor-dependent glycosyltransferases, sulfotransferases, sulfatases, and heparanases (6, 45). The presence and amount of these GAG-related enzymes help identify high-risk tumors and develop targeting therapies (46). In colon tumors, for example, significant upregulation of extracellular sulfatases SULF-1/2 has been observed and may indicate general alteration of HS 6-O-sulfation patterns in colon tumors (47).

As discussed in the introduction, hundreds of different extracellular regulatory proteins, such as growth factors, chemokines, and morphogens, also involved in cancer, interact with the GAG portion of HSPG, requiring specific glycosides sequences and sulfation patterns (23).

The peculiar post-translationally regulated variability of HSPG has made it difficult to study their activity in cancer cell biology.

NT4 is already known to have major effects on cancer cells, such as inhibition of migration and invasion of ECM induced by FGF (20).

We examined the expression of syndecans and glypicans in a panel of cancer cell lines that NT4 binds. The binding affinity of NT4 with human rHSPG expressed by these cells was then analyzed by SPR. NT4 did not bind syndecan-3, but it bound glypican-3 and -4, and also syndecan-4, but with one fifth of the affinity shown for glypicans.

TABLE 1 | Kon, koff, and KD of recombinant glypicans and syndecans and oligosaccharides.

	Ka ($M^{-1}s^{-1}$)	Kd (s^{-1})	KD (M)
Syndecan-4	0.901E+4	23.16E−4	2.570E−7
Glypican-3	2.392E+4	14.33E−4	5.989E−8
Glypican-4	0.871E+4	6.719E−4	7.708E−8
S06a	0.106E+4	25.75E−4	2.427E−6
S06b	0.149E+4	40.39E−4	2.700E−6
S12	0.353E+4	9.126E−4	2.578E−7

Glypicans and syndecans have different GAG chains: glypicans only carry HS chains, whereas syndecans-2 and 4 have HS chains and syndecans-1 and 3 have HS and CS chains (4, 48). Besides, HS posttranslational modifications occur in clusters, i.e., HS has some domains that are more densely sulfated than others. For example, the FGF binding domain that has 2-, 6-, and N-sulfation, carries seven sulfated groups in five residues, whereas the anti-thrombin binding domain contains six sulfated groups in five residues. In contrast, CS has more homogeneously sulfated patterns with long tracts carrying an average of four sulfates every five residues (49).

The NT4 affinity profile is therefore consistent with our previous results that showed a preference of the peptide for HS chains featuring patches of dense sulfation, compared to CS (49).

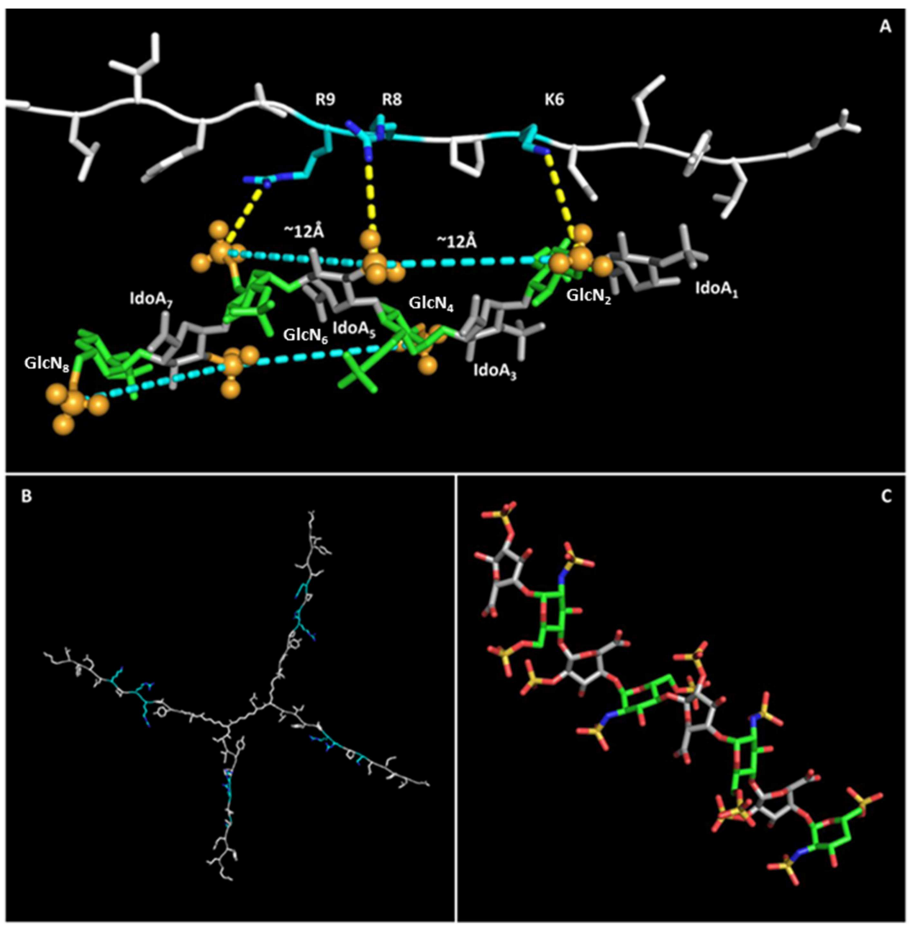

FIGURE 5 | NT4-sulfated oligosaccharide hypothetical complex. **(A)** Model of NT4 complexed with the 8-mer sulfated oligosaccharide. Clusters of sulfates on both sides of the helical structure of the oligosaccharide are identified by pale blue lines, with the sulfates involved represented as spheres. Polar interactions between the positive charges on peptide residues and sulfate negative clusters are drawn as dashed yellow lines. **(B)** Model of NT4 structure with KPRR motifs in pale blue. **(C)** Structure of the sulfated oligosaccharide (29).

Another important finding regarding NT4 recognition of sulfated GAG chains came from the analysis of sulfatase expression in the same panel of human cancer cell lines. NT4 binding to the cancer cell membrane was inversely correlated with expression of sulfatases. NT4 binding was higher in cell lines with lower expression of sulfatases, particularly SULF-1, i.e., HT-29 and PANC-1, confirming the determinant role of 6-O-sulfate groups for recognition by NT4.

Using 8-mer and 9-mer HS oligosaccharides with analog disaccharide composition and different sulfation sites, a possible recognition motif was identified that includes repeated 6-O-sulfates alternating with N- and/or 2-O-sulfates. This finding is again consistent with the preference of NT4 for HS more than for CS. CS carries GAG chains with 2-O-sulfates and 4-O-sulfates, whereas HS has 6-O-sulfates alternating with N- or 2-O-sulfates.

The possible structure of the NT4-sulfated oligosaccharide complex was then reconstructed by molecular modeling, taking into account our information on amino acids in NT4 sequences, i.e., KPRR, previously demonstrated to be essential for heparin and HS binding (13, 19). The modeling showed that the distance between the crucial positive residues of NT4 is completely compatible with ionic interaction with sulfates on the oligosaccharide. Moreover, assuming a helical structure of the oligosaccharide, which is considered usual for sulfated oligosaccharides, sulfate groups lying on opposite sides of the helix can interact with positive residues on two peptide sequences of the branched structure, thus favoring multivalent binding, and explaining the high affinity and selectivity of NT4 for highly sulfated GAGs. Being a branched peptide, NT4 can give multiple binding to repeated domains on the same GAG chain or on different GAG chains of the same HSPG, improving binding affinity. Specificity of GAG ligand binding, which allows formation of the GAG–ligand–receptor complex that triggers signal transduction, is mediated by multivalent electrostatic interactions between GAGs and growth factors or proteins of the ECM. The presence of binding sites of growth factors and proteins on GAG chains is no longer disputed, and the exact structure and motifs of the recognition patterns are being explored (23, 50, 51).

NT4 and possibly newly selected branched peptides can be designed and used to unravel the exact structure of binding sites on GAG chains. These tools will be essential probes for reconstructing binding sites for cancer-involved ligands on GAGs, paving the way for new cancer detection and treatment options.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

CF, JB, LD, APi, APa, and LB conceived and designed the experiments. AB designed and performed the modeling experiments. EK performed the qRT PCR experiments. JB, GR, EM, and LD performed flow cytometry, SPR, and Western blot. CF and LB wrote the paper. CF supervised the project. All authors read and approved the final manuscript.

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REVIEW
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Heparanase: A Challenging Cancer Drug Target

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Heparanase has been viewed as a promising anti-cancer drug target for almost two decades, but no anti-heparanase therapy has yet reached the clinic. This endoglycosidase is highly expressed in a variety of malignancies, and its high expression is associated with greater tumor size, more metastases, and a poor prognosis. It was first described as an enzyme cleaving heparan sulfate chains of proteoglycans located in extracellular matrices and on cell surfaces, but this is not its only function. It is a multi-functional protein with activities that are enzymatic and non-enzymatic and which take place both outside of the cell and intracellularly. Knowledge of the crystal structure of heparanase has assisted the interpretation of earlier structure-function studies as well as in the design of potential anti-heparanase agents. This review re-examines the various functions of heparanase in light of the structural data. The functions of the heparanase variant, T5, and structure and functions of heparanase-2 are also examined as these heparanase related, but non-enzymatic, proteins are likely to influence the *in vivo* efficacy of anti-heparanase drugs. The anti-heparanase drugs currently under development predominately focus on inhibiting the enzymatic activity of heparanase, which, in the absence of inhibitors with high clinical efficacy, prompts a discussion of whether this is the best approach. The diversity of outcomes attributed to heparanase and the difficulties of unequivocally determining which of these are due to its enzymatic activity is also discussed and leads us to the conclusion that heparanase is a valid, but challenging drug target for cancer.

Keywords: heparanase, heparan sulfate, heparin, drug discovery, cancer, tumor progression, heparanase-2

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INTRODUCTION

Heparanase is a heparin/heparan sulfate (HS) specific endo-β-D glucuronidase. It was originally called a heparan sulfate degrading endoglycosidase, heparitinase or heparinase, and the human enzyme was first isolated from placenta (1) and later from platelets (2), whereas the mouse enzyme was initially isolated from a murine mastocytoma (3). In the mid-late 1980s, we and others demonstrated that the ability of heparin to inhibit metastasis was consistent with its ability to inhibit heparanase released by tumor cells, and this activity was independent of heparin's anticoagulant activity (4, 5). This finding firmly placed heparanase as cancer-associated enzyme. In 1999 the cloning of heparanase from human platelets, and the discovery that the sequences of heparanase from human activated murine T lymphocytes and rat adenocarcinoma cells were highly homologous, confirmed that the heparanase activity detected in normal mammalian cells

and the tumor enzyme are the same (6). Another 1999 study published back-to-back with the above paper, also reported the cloning of human heparanase, and here it was reported the heparanase gene was preferentially expressed in tumor tissue compared to normal tissue (7). They found when poorly metastatic murine melanoma and T-lymphoma cells were transfected with the heparanase gene, this resulted in a massive increase in metastases (7). These papers led to a plethora of studies focusing on the development of heparanase inhibitors as anti-cancer agents. Four of these molecules: PI-88 (muparfostat), PG545 (pixatimod), SST0001 (roneparstat), and M-402 (necuparanib) have progressed to clinical trials. Despite impressive effects in a variety of preclinical models, the clinical data have been less convincing, and development of some of these compounds has discontinued or has stalled (8).

As has been highlighted in recent reviews, it is now known that heparanase is involved in a range of pathologies in addition to cancer, including inflammation, diabetes, bone necrosis, liver fibrosis, amyloidosis and Alzheimer's disease, and in the infection and spread of numerous viruses (9–12). The contributions of this protein to normal physiological and disease processes are complex, particularly given that heparanase has been reported to act in enzymatic and non-enzymatic ways (9, 13, 14). The possibility that heparanase possesses more than one functional site: a catalytic site and at least one other functional site, complicates the interpretation of heparanase gene knock-out studies and may complicate the development of heparanase inhibitors. Although there is no evidence for more than one enzymatically active mammalian heparanase, a second closely related protein called heparanase-2, or Hpa2, was cloned in 2000 (15). In contrast to heparanase, Hpa2 lacks catalytic activity (16). However, like heparanase, it has a role in cancer, but generally in tumor suppression, not tumor promotion as is seen with the catalytic protein (17). In adults, heparanase is also involved in normal physiological processes like tissue regeneration and repair, wound healing, hair growth, dendritic cell migration, and the implantation of embryos during the early stages of pregnancy (18, 19).

While this manuscript was in preparation an extensive review on heparanase in cancer highlighting the numerous synthetic and chemically modified natural compounds that have been produced as potential drugs targeting heparanase was published (20), accordingly we will take a different approach here. In this article, we look at heparanase from the viewpoint of heparanase as a drug target, and ask why has the translation from preclinical studies to successful clinical trials of drug candidates targeting heparanase in cancer been so difficult?

Heparanase Structure

Heparanase is produced as a preproenzyme; the signal sequence is removed upon entry into the endoplasmic reticulum (ER) giving rise to a 65 kDa latent proenzyme which is secreted. At the cell membrane the proenzyme binds to HS-proteoglycans and of these, the syndecans, are particularly important for complexing with the enzyme, then internalizing and transporting it to the late endosomes or lysosomes where the proenzyme is processed (21). Heparanase can also be internalized by a HS independent mechanism by binding to mannose-6-phosphate receptors on the cell surface (22). Purification of active platelet heparanase revealed the active enzyme is a non-covalently linked heterodimer comprising the N-terminal 8 kDa fragment, and the 50 kDa C-terminal fragment of proheparanase; therefore, processing resulted in the excision of an internal linking segment (23). Sequence alignment and folding prediction studies suggested heparanase adopts a $(\beta/\alpha)_8$-TIM barrel fold (eight alternating β-strands and α-helices), a motif common in glycosidases. In heparanase, both fragments were predicted to contribute to the TIM barrel fold, with a β/α/β element coming from the 8 kDa fragment and the remaining alternating β-strands and α-helices coming from the 50 kDa fragment (24).

The crystal structure of human heparanase has verified these predictions (25). A baculovirus dual-expression system was used to produce the active enzyme for the crystal structure. The cDNAs encoding either the 8 kDa fragment or the 50 kDa fragment were both placed into a single bacmid, but under the control of different viral promotors, this enabled the two fragments to co-translationally fold into the mature enzyme. The crystal structure also confirmed the $(\beta/\alpha)_8$-TIM barrel fold is flanked by a smaller COOH-terminal β-sandwich domain (C-domain) (**Figure 1**), which had been described previously from a predicted three-dimensional structure (26). The C-terminal region includes a hydrophobic and conserved sequence (residues 515–534) (27) and a disulfide bond between Cys^{437} and Cys^{542} (**Figure 1**), which is required for the activation and secretion of heparanase (28). Site-directed mutagenesis confirmed that the catalytic mechanism uses a proton donor (Glu^{225}) and a nucleophile (Glu^{343}), and consistent with the active sites of other glycosyl hydrolases, these two amino acids are essential for the catalytic activity of heparanase (29). The crystal structure revealed these two residues are in a cleft ∼10 Å long located in the $(\beta/\alpha)_8$ domain, and lined with the basic side chains of arginines and lysines (25). The crystal structure of proheparanase confirmed that the 6 kDa linker, which is excised in native, active heparanase, forms a helix which is located immediately above the active site cleft sterically blocking all but a small pocket containing the catalytic residues. Although the linker region sterically blocks the access of bulky HS substrates to the cleft, it may be a binding site for smaller endogenous substrates the nature(s) of which are unknown (30). These structural data indicated that the internalization of proheparanase that occurs though binding to cell surface HS-proteoglycans, must occur via an HS binding site that is distinct from the catalytic cleft. It is unclear as to what region of heparanase is involved at the cell surface in HS-independent internalization. Although specific amino acids within the hydrophobic region of the C-terminal region of heparanase were found to be required for heparanase activation, the data are consistent with a role in heparanase intracellular trafficking and secretion rather than in binding to a cell surface protein (27).

The structure of heparanase in complex with HS analogs or with a heparin tetrasaccharide was determined from crystallization studies at pH 5.5, the optimal pH for heparanase

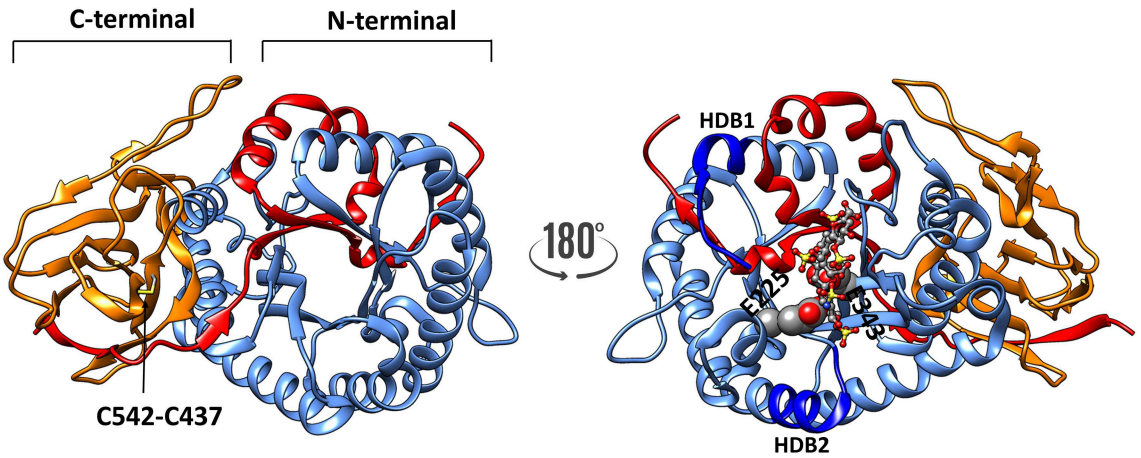

FIGURE 1 | The three-dimensional structure of heparanase (PDB code: 5e9c) showing the 8 kDa unit as red ribbon, whereas the catalytic (TIM barrel) is shown in blue. The C-terminal 413–543 shown in orange ribbon. There is a disulphide bond C437-542 (indicated by an arrow) present in both activated heparanase and proheparanase. The disulphide bond and heparin tetrasaccharide are shown in sticks representation, whereas the catalytic residues are shown as spheres, respectively. HBD1 and HBD2 (dark blue) refer to heparin binding sites consisting of residues 158–171 and 270–280, respectively.

enzymatic activity. These data revealed the nature of the bonds that retain the HS substrate in the cleft. Interestingly, these are predominately hydrogen bonds. There is a network of direct hydrogen bonds linking the tetrasaccharide substrate, which is the smallest HS or heparin fragment cleaved, with non-basic amino acids at the base of the cleft (**Figure 2**). These amino acids are Gly^{389}, Asn^{64}, Tyr^{391}, Gly^{349}, Gly^{350}, Thr^{97}, Asn^{224}, Gln^{270}, and Asp^{62}. In contrast, only three amino acids appear to be involved in electrostatic interactions, and these are Lys^{159}, Arg^{272}, and Arg^{303} (25, 31). Earlier reports where homology modeling was used to predict the amino acids interacting with the HS substrate suggested electrostatic interactions with basic residues made a greater contribution (32, 33). We have examined heparanase in four different animal species to determine whether the critical amino acids for substrate binding in the human enzyme are conserved. This study revealed that the catalytic residues are conserved in the species examined, as were the amino acids involved in hydrogen bonding except for Asn^{64} and Thr^{97}, and of the residues involved in electrostatic interactions only Lys^{159} was not conserved (31). In contrast, the amino acids surrounding the HS binding residues were much less conserved. Thus, it is likely that the positively charged amino acids serve to direct the HS chain into the catalytic cleft, but hydrogen bonding is of key importance for stable substrate binding.

Heparanase Substrate Recognition

The substrate specificity of heparanase has been extensively investigated. A study using a series of structurally defined oligosaccharides ranging in size from penta- to nona-saccharides where the heparanase cleavage products were examined by electrospray ionization mass spectrometry (ESI-MS), revealed that heparanase most favors cleaving the linkage of a glucuronic acid linked to 6*O*-sulfated glucosamine that may be *N*-sulfated or *N*-acetylated (34, 35). Heparanase was found to be a strict endo-β-glucuronidase, only cleaving internal linkages. In fragments of repeating –GlcUA-GlcNS6S/GlcNAc6S- not every –GlcUA-GlcNS6S- linkage is cleaved, rather consecutive linkages of this structure are cleaved when GlcNAc6S is a component of the non-reducing end, trisaccharide domain, which is not cleaved. However, if this residue is replaced with GlcNS6S, the immediate next cleavage site is skipped to produce a bigger fragment from the middle portion of the substrate (34). Molecular modeling of heparanase co-crystallized with tetrasaccharides from heparin revealed why a GlcUA2S or an IdoUA2S is not part of the cleavage site; steric clashes occur between heparanase Asn^{224} and the bulky 2*O*-sulfate (25). Moreover, when heparin type ligands containing IdoUA bind to heparanase the IdoUA residues are constrained in the $^2S_0$ conformation and cannot undergo the conformational changes that are required for cleavage (25, 36), and so they act as competitive inhibitors of HS cleavage by heparanase. This study also revealed why the favored trisaccharide for cleavage has an *N*-sulfated glucosamine in the −2 position and 6*O*-sulfated glucosamine in the +1 position. The binding of heparanase introduces a bend across −2, −1, +1 of the trisaccharide, and the bend separates the 2*N* and 6*O* sulfates allowing the catalytic residues of heparanase to more easily access the anomeric center of the −1 GlcUA and so catalyse cleavage (25). In this context, the 2*N* and 6*O* sulfates have been described as "mechanistic handles" that heparanase uses to open the HS helix, as well as acting as a recognition signal to direct the enzyme to particular glycan structures in the HS chain that are permissible for cleavage (25).

FUNCTIONS OF HEPARANASE IN CANCER BIOLOGY

Extracellular Heparanase Functions Associated With Enzymatic Activity

The enzymatic function of heparanase is important both within the cell and extracellularly. Extracellularly heparanase is one

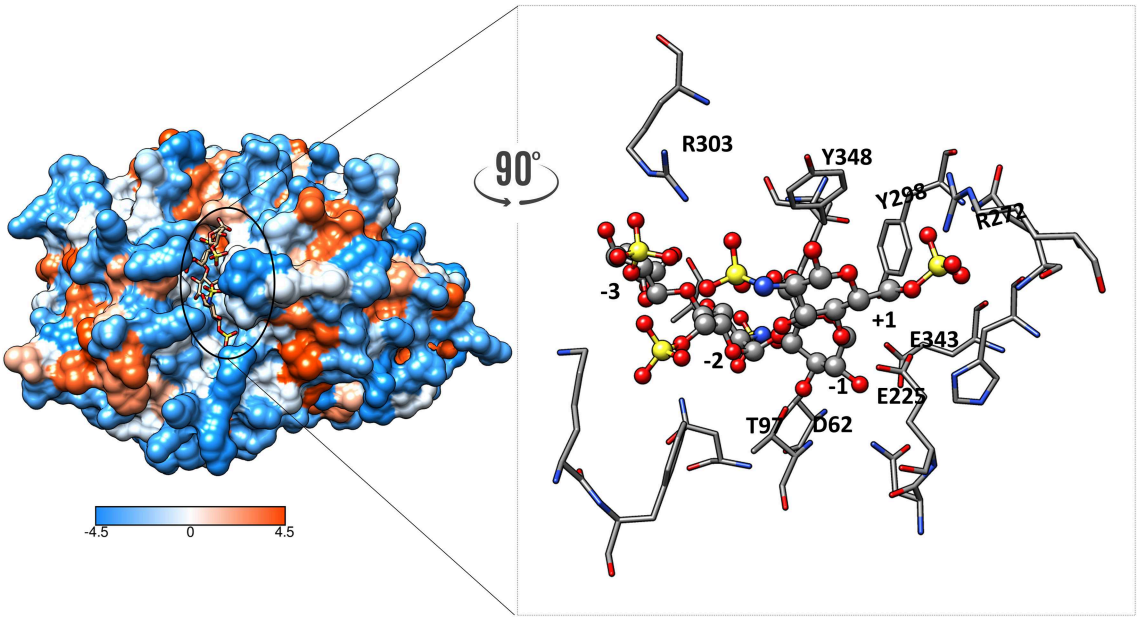

FIGURE 2 | Hydrophobic surface of heparanase showing heparin tetrasaccharide in the catalytic cleft. The orange color indicates hydrophobicity whereas the blue show hydrophilic surfaces. Zoom-in view of the catalytic cleft in complex with the heparin tetrasaccharide substrate showing interactions at various subsites. Hydrogens are not shown for clarity.

of an array of enzymes that act on the extracellular matrix and basement membrane surrounding a primary tumor to weaken these structures and so facilitate tumor cell invasion into the surrounding tissues and assist metastasis formation. Experimental heparanase overexpression in a transgenic mouse model verified extensive HS fragmentation *in vivo* (37). The fragmentation of HS chains was the first appreciated function of this protein, and it remains the key heparanase function assayed by the majority of drug discovery programs targeting heparanase (5, 20, 38). The degradation of HS chains on matrix proteoglycans by heparanase, as well as dissolving the physical barrier, also releases latent growth factors, like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF2), hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), and keratinocyte growth factor (FGF4) which are bound and sequestered to the matrix by HS (20). Cleavage of HS gives these signaling molecules better access to their receptors. Indeed, FGF2 signaling is enhanced by moderate heparanase activity possibly because the formation of FGF2-FGFR-HS complexes necessary for sustained signaling is facilitated (39). Also, heparanase overexpression has been shown to produce HS structures that more readily facilitate FGF and FGF receptor complex formation than HS structures obtained when heparanase is not overexpressed. This is probably because 6*O*-sulfotransferase, an enzyme involved in HS and heparin biosynthesis, is upregulated in heparanase overexpressing tissue, resulting in increased 6*O*-sulfation of HS (40). The notion that heparanase, through its enzymatic activity, releases sequestered pro-angiogenic growth factors like VEGF, and so facilitates VEGF receptor interactions and angiogenesis in tumor models has been demonstrated (41). In addition, there are examples where heparanase gene silencing has resulted in tumors that are less vascularised and less metastatic than their controls (42, 43), all of which very strongly point a contribution of heparanase in tumor angiogenesis.

Heparanase degrades HS chains on cell surface proteoglycans as well as on proteoglycans within the extracellular matrix. The cell surface proteoglycan that has received the most attention in this regard is syndecan-1. This proteoglycan potentially has two HS chains and three chondroitin sulfate chains covalently linked to its extracellular domain. The trimming of these HS chains by heparanase exposes a site on the syndecan-1 core protein that is susceptible to cleavage by proteases, causing shedding of the extracellular domain of syndecan-1 from the cell surface (44). The shed syndecan can bind to endothelial cells or tumor cells via the VEGF receptor, VEGFR2, and/or the integrin α4β1, to cross-link these two proteins. This causes the activation of VEGFR2 and the trafficking of the α4β1-VEGFR2 complex to the leading edge of the cell where it facilitates tumor, or endothelial cell, migration via Rac1 (Ras-related C3 botulinum toxin substrate 1) activation and the reorganization of the actin cytoskeleton (45). This mechanism [reviewed in (46)] may explain how heparanase's enzymatic activity can enhance both tumor cell invasion and angiogenesis.

Heparanase (either active or latent) has been reported to cluster syndecans on the cell surface thereby enhancing cell spreading and adhesion. The mechanism for these effects involves Rac1 activation in a process that does not require heparanase enzymatic activity (47). Studies with heparanase, and a disulfide-linked heparanase peptide dimer (Lys^{158}-Asp^{171}Cys x2), that contains an amino acid sequence that binds heparin/HS

(48), were interpreted as demonstrating that heparanase cross-links or clusters cell surface syndecans to facilitate cell adhesion and spreading, processes mediated by PKCα (protein kinase C alpha), Rac1, and Src (a proto-oncogene tyrosine-protein kinase) (49). The peptide dimer was more potent than intact heparanase in these assays, but unlike heparanase-syndecan complexes which were internalized, the peptide dimer-syndecan complexes remained on the cell surface. Data from the crystal structure of heparanase, indicated that the two HS binding sites proposed from the sequence, Lys^{158}-Asp^{171} and Gln^{270}-Lys^{280} are located at either end of the HS binding cleft (25, 48), and our analysis of the crystal structure indicated that the Gln^{270}-Lys^{280} region is of major importance for HS binding in the intact proheparanase and also in heparanase. A third potential HS binding region identified from the heparanase sequence, Lys^{411}-Arg^{432} and which includes the motif KRRKLR (48), appears not to be involved in HS binding as in the folded protein this motif is largely buried (25). When expressed as peptides, it was reported that the Lys^{158}-Asp^{171} peptide exhibited a higher affinity for heparin/HS than the Gln^{270}-Lys^{280} peptide (48), which is in contrast to our findings using the crystal structures of both activated heparanase and proheparanase. Indeed, from our analyses, it appears as if there is a single HS binding region that includes Lys^{158} and Lys^{159} along with the Gln^{270}-Lys^{280} region. Thus, it is possible that the peptide dimer may not be a good model for heparanase and hence may not mirror the behavior of heparanase in these assays. Nevertheless, both enzymatic and non-enzymatic functions of heparanase are probably involved in cell invasion and in the pro-angiogenic activity of this enzyme (10, 50), and it is likely that syndecan clusters are involved, but whether heparanase, on its own, clusters syndecan is questionable from these data.

The HS fragments produced as a result of heparanase cleavage can have signaling activities in their own right. For example, heparanase was shown to stimulate the release of pro-inflammatory cytokines [interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α] from peripheral blood mononuclear cells with the mechanism being attributed, in part, to the binding of HS fragments, released by heparanase cleavage, to Toll-like receptor-4 (TLR4); thereby stimulating signaling through the MyD88 (myeloid differentiation primary response 88) pathway (51). However, heparanase also appears to influence cytokine secretion from immune cells in the absence of enzymatic activity. Heparanase caused activated T cells to shift from a T-helper (Th)1 profile to a more Th2 profile as indicated by the cytokines released by activated splenic lymphocytes, there being decreased levels of IL-12 and TNF-α and increased levels of IL-4, IL-6 and IL-10 released, an effect that was independent of its enzymatic activity (52). Interestingly, macrophages isolated from heparanase knock-out mice expressed lower levels of various inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-10, IL-1β, and CXCL2 (also called macrophage inflammatory protein 2-alpha) (53). These heparanase negative macrophages also displayed diminished migration and phagocytic capacity compared to wild type (WT)-macrophages, suggesting that heparanase is important for macrophage activation and function. This study also showed that heparanase negative macrophages, in contrast to WT-macrophages, could not invade tumor tissue in response to CXCL2 and did not attenuate tumor growth (53). In another study using gene-modified mice that lacked heparanase in natural killer (NK) cells, it was demonstrated that endogenous NK cell heparanase was required for effective tumor NK cell invasion and immune-surveillance. The data suggested that heparanase deficient NK cells were unable to degrade the tumor extracellular matrix and invade the tumor. As a result, the mice were susceptible to tumor growth following inoculation with cell lines of prostate or mammary carcinoma, metastatic melanoma, or lymphoma (54). These two examples indicate that heparanase can be beneficial for combatting tumor growth, as well as detrimental; the latter being more commonly highlighted. The contribution of heparanase to leukocyte behavior and tumor progression is discussed in detail elsewhere in a recent review (55), where it is made clear that heparanase can enhance tumor clearance by facilitating immune cell infiltration of tumors, as well as promote tumor survival by facilitating tumor cell migration.

Extracellular Heparanase: Non-enzymatic Activities

Several cell adhesion pathways are regulated, or mediated by heparanase in a manner that does not involve enzymatic activity. For example, heparanase was shown to induce the clustering of breast cancer cells in a manner reminiscent of circulating tumor cells, and knockdown of heparanase inhibited the formation of these cancer cell clusters and suppressed breast cancer metastasis (56). Heparanase has also been shown to augment angiogenesis by a mechanism that involves activation of the β1 integrin/hypoxia-inducible factor (HIF)-2α/fetal liver kinase 1 (Flk-1)/P38 MAP kinase/heat shock protein (HSP)27 adhesion and signaling pathway, a mechanism that does not involve enzymatic activity or the release of cytokines by HS degradation (57). Another non-enzymatic function of heparanase is the activation of Akt (a protein kinase with roles in apoptosis, cell migration, and proliferation), most likely by a mechanism involving cross-talk between a putative heparanase cell surface receptor and integrins (58). Data were reported, which suggested that heparanase stimulation activates PI3-kinase (PI3K) which then phosphorylates Akt. Integrins were found to promote the activation of the PI3K-Akt pathway by heparanase, via a mechanism that involves focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK2) auto-phosphorylation. It was also shown that exposure to heparanase increased resistance to stress-induced apoptosis, and this was assumed to involve the PI3K-Akt pathway, but this was not proven (58).

Heparanase has been shown to have a direct effect on the coagulation system via mechanisms that are independent of enzymatic activity. In humans, both the 50 kDa (active) and 65 kDa (latent) forms of heparanase are abundant in platelets and are released following the interaction of thrombin with the protease-activated receptor (PAR)-1 on the platelet surface leading to platelet activation. A similar mechanism was shown to release heparanase from granulocytes (59). Heparanase from these sources forms a complex with tissue factor (TF) which

leads to increased factor Xa levels and procoagulant activity. This effect is regulated by tissue factor pathway inhibitor-2 (TFPI-2) binding heparanase and inhibiting the TF/heparanase complex. Heparin also inhibits the interaction between TF and heparanase. The region on heparanase, which was identified from a collection of heparanase peptides as involved in procoagulation and TF binding, spans amino acids 423–438. This region was described as a weak heparin binding domain and is not involved in the catalytic function of heparanase (13, 60). However, this region is largely buried in the intact protein (25), raising the question as to whether, or which part of, the region spanning amino acids 423–438 is involved in TF binding in the intact protein. Interestingly, peptides from TFPI-2 that inhibited the procoagulant activity of heparanase, but not the catalytic activity, also reduced tumor growth and vascularisation in murine tumor models (61). It is likely that platelet heparanase contributes to platelet-endothelial cell adhesion and the hyper-thrombotic conditions seen in cancer patients via another mechanism. This mechanism involves P-selectin, on activated platelets, binding HS on endothelial cells that have been trimmed by heparanase (62). A potential regulator of heparanase released into the circulation from platelets and leukocytes is the relatively abundant plasma protein, histidine rich glycoprotein (HRG). HRG interacts directly with heparanase to enhance its enzymatic activity, and this interaction is inhibited by HS. Heparanase binding to cell surfaces is also partially inhibited by HRG, and it was suggested this had the effect of maintaining heparanase in a soluble extracellular form rather than it being internalized into cell endosomes (63).

Intracellular Heparanase

Intracellularly heparanase is primarily located in lysosomes or late endosomes where the acidic environment favors enzymatic activity, although some intracellular heparanase was found to reside in autophagosomes. Further evidence from heparanase knockout mice indicated heparanase might modulate autophagy (64), suggesting it has a role in the normal physiology of lysosomes. In the late endosomes, heparanase has been shown to trim the HS chains of syndecans (65), whilst in mast cells, heparanase is sorted to the secretory granules where it cleaves heparin chains covalently attached to serglycin (66). The HS or heparin fragments produced by heparanase cleavage range in size from 5 to 20 kDa indicating heparanase regulates the size of the free HS/heparin chains secreted from mast cells rather than causing the total degradation of HS/heparin chains.

Heparanase activity in the late endosomes has an outcome other than the fragmentation of HS chains. It regulates exosome excretion and the composition of proteins within secreted vesicles so that cells with high levels of heparanase release more exosomes and these vesicles contain higher levels of HS binding growth factors like VEGF and hepatocyte growth factor (HGF) as well as syndecan-1 (67). Moreover, the effects of heparanase on exosome secretion were dependent on HS cleavage, and the production in endosomes of syndecans with trimmed HS chains that can undergo proteolytic cleavage to generate syndecan C-terminal fragments that remain within the membrane, and cluster into microdomains. By this means, heparanase is a key modulator of exosome biogenesis that occurs by the syndecan-syntenin-ALIX (a regulator of the endo-lysosomal system) pathway (65, 68). An issue that remains unresolved is to what extent the cargo contained in these exosomes is responsible for the effects attributed to heparanase in sustaining tumor growth, tumor cell invasion, and the augmentation of angiogenesis, rather than heparanase itself. Most interestingly, the exposure of myeloma cells to various chemotherapy drugs dramatically enhanced the secretion of exosomes containing high levels of the 65 kDa form of heparanase as a cargo (69). The interaction of macrophages with these exosomes caused enhanced macrophage migration and secretion of TNF-α, a myeloma growth promoting cytokine. The latent heparanase was located on the surface of these exosomes and was readily taken up by tumor cells and activated, giving it the ability to modify the tumor microenvironment and so facilitate disease progression (69).

A significant fraction of heparanase is located in the nucleus. In glioma and breast carcinoma, this was found to be about 7% of the cytosolic enzyme, and nuclear heparanase was enzymatically active, degrading nuclear HS (70). Indeed, heparanase was found to co-localize with syndecan-1 in the nucleus of mesenchymal tumors (71). Curiously, given the association of heparanase with cancer, the translocation of heparanase into the nucleus has been associated with cell differentiation; the differentiation of HL-60 cells into monocytes and macrophages (72), and the differentiation of, and expression of differentiation markers by esophageal keratinocytes (73) being two examples. Possibly the intracellular location of heparanase may be a prognostic indicator for some cancers. In squamous cell carcinoma of the head and neck, nuclear localization of heparanase predicted a good outcome, whereas cytoplasmic heparanase correlated with a poor prognosis (74). More specifically, nucleolar and nuclear heparanase were proposed to have contrasting effects on cell proliferation. Nuclear heparanase was reported to be involved in differentiation, but active nucleolar heparanase was said to augment cell proliferation via its modulation of DNA topoisomerase I activity, an enzyme essential for DNA replication and gene transcription that is inactivated by HS (74). Other studies suggested that heparanase can bind DNA and/or chromatin. One study demonstrated, using atomic force microscopy, that heparanase bound plasmid DNA, most probably by a charge mediated interaction (75). Another study found evidence that heparanase (likely the ∼50 kDa form) belongs to a group of chromatin-associated proteins that can modulate gene transcription. More specifically, using T lymphocytes, heparanase was found to preferentially associate with euchromatin. It was associated with the promoters and 5′ coding regions of a large number of different genes and was part of an active transcription complex necessary for the transcription of a subset of inducible immune response genes. The evidence further suggested that heparanase acts by binding LSD1, a lysine specific demethylase, thereby preventing recruitment of MLL, a histone methylase, resulting in modification of histone H3 methylation patterns to one associated with inducible gene transcription (76). Such effects are due to the binding capability of heparanase rather than its catalytic activity.

If heparanase has the potential to alter gene transcription either by directly binding DNA or indirectly by regulating methylation patterns, cancer cells with high levels of endogenous heparanase should express a different pattern of genes than cells with low or normal heparanase levels. This was found to be the case in melanoma cells subjected to siRNA mediated knock-down of heparanase expression. The genes down-regulated by heparanase silencing were all categorized as nucleosome genes or nucleosome assembly genes, whereas numerous pro-apoptotic genes were up-regulated following heparanase silencing. Moreover, a significant increase in apoptosis was detected in the absence of heparanase that involved the caspase 3/poly(ADP-ribose) polymerase-1 (PARP-1) pathway, as revealed by the appearance of fragmented caspase 3 and PARP-1 (77). This study suggested that heparanase may directly interfere with apoptotic pathways, and so could protect cancer cells from apoptosis during therapy. Clearly, more work is required to unravel the complexity of heparanase's activities in the nucleus.

SPLICE VARIANTS OF HEPARANASE

In addition to full-length heparanase alternatively spliced variants of human heparanase have been detected. A splice variant lacking exon 5 was detected in cDNA from a renal cell carcinoma infiltrated kidney. The reading frame of the wild-type gene was conserved in this variant, resulting in a protein lacking 58 amino acids, including the active site proton donor Glu^{225} (78). Very little is known about this variant; but from the reported study, it did not appear to be secreted, it is not cleaved, and it lacks enzymatic activity. Another variant termed T5, has attracted more attention. This truncated, enzymatically inactive heparanase variant arises when 144 base pairs of intron 5 are joined with exon 4, giving rise to a protein of between 15 and 17 kDa, depending on its glycosylation status (79). The T5 protein does not bind heparin (79), probably because it contains only a few of the amino acids identified from the crystal structure of heparanase that are involved in binding heparin (25). In addition, its structure may not allow heparin binding because it is likely T5 only partially resembles active full-length heparanase in its three-dimensional conformation. This conclusion is supported by monoclonal antibody (mAb) data which suggests that in T5 the linker region is exposed on the outside of the protein and is strongly immunogenic (80), whereas the linker region is excised in active heparanase.

Like heparanase, T5 significantly enhanced tumor development in a myeloma xenograft model, despite its inability to degrade HS (79). Interestingly, the effect of both T5 and heparanase in this model was not apparent until 3 weeks post subcutaneous inoculation when the development of tumor xenografts expressing either T5 or heparanase was markedly enhanced compared to controls lacking these proteins. Both the density of blood vessels and vessel maturation were enhanced with T5 or heparanase expression, but T5 expression better-allowed pericyte coverage of blood vessels and small capillaries (79). *In vitro* studies indicated that expression of these proteins increased the proliferation of myeloma cells and enhanced colony formation in soft agar of myeloma, pharynx carcinoma, and human embryonic kidney cells. Human embryonic kidney cells and myeloma cells overexpressing T5, or heparanase, displayed enhanced Src phosphorylation, whereas Erk (extracellular signal regulated kinase) phosphorylation was not affected, and in the case of T5 this effect was independent of HS. The use of Src inhibitors caused colony formation in T5 and heparanase expressing cells to resemble that of control cells, which led the authors to conclude that Src activation contributes to the enhanced cell proliferation seen with T5 or heparanase expression. Finally, a cohort of renal cell carcinoma specimens was examined for both T5 and heparanase mRNA and were immunostained with a mAb that preferentially recognizes T5 over heparanase. These data indicated that the intensity of T5 staining was positively associated with tumor size and grade, and when T5 mRNA was detected so to was heparanase mRNA (79, 80). A very recent study using glioma revealed that T5, like heparanase, was associated with the upregulation of CD24, a protein that is significantly associated with malignancy and poor outcomes in a number of carcinomas (81). Thus, the T5 variant of heparanase displays many of the biological effects of the full-length protein, at least in the cancer models studied to date, yet it lacks enzymatic activity.

HEPARANASE-2 (HPA2)

Structure and Biochemistry

The publication of the sequence of heparanase prompted two groups to use this sequence to search EST databases to determine if mammalian heparanase was one of a family of enzymes. From this work another heparanase, Hpa2 was identified and cloned (15, 19). Both groups found that alternative splicing produced different variants of Hpa2; McKenzie et al. reported three variants (15), whereas Vreys and David as well as finding the earlier variants, reported a fourth splice variant, hep-2B (19). Of these variants, hep2c (corresponding to hep-2AB from Vreys and David) and hep-2B are secreted, and both have been reported to bind to HS-proteoglycans on cell surfaces (16, 19). However, it is the former of these variants, hep2c/hep-2AB, which has become synonymous with Hpa2 and hence in our discussion, we will be referring to hep2c/hep-AB.

Not surprisingly given the means by which Hpa2 was identified there is a high degree of similarity between heparanase and Hpa2 with the overall identity by sequence alignment being 42–44%, including conservation of the two catalytic residues of heparanase, Glu^{225} and Glu^{343} (16, 19). The homology between heparanase and Hpa2 is most apparent in the 8 kDa N-terminal, and the 50 kDa C-terminal portions of heparanase that result from cathepsin L processing. The linker region that is excised in heparanase is far less conserved (15, 19). Importantly, the amino acid site (Gln^{157}-Lys^{158}) that is first cleaved by cathepsin L, including the essential Tyr^{156}, is not conserved in Hpa2 and is replaced by Phe^{194}, Ser^{195}, Asn^{196} (19). Moreover, there is no biochemical evidence for similar post-translational processing of

Hpa2 as is seen with heparanase (16, 19), which is consistent with its lack of catalytic activity.

Unfortunately, the published molecular model of Hpa2 is of the region corresponding to the 50 kDa fragment of heparanase, it, therefore, lacks the linker region and the *N*-terminal region (82). Also, it predates the crystal structure of human heparanase (25). We have developed a homology model of Hpa2 using the SWISS-MODEL server and proheparanase as the template [PDB 5LA4 (30)]. In this model, the linker domain in Hpa2 partially occupies the catalytic cleft (**Figure 3**) and is likely to obscure at least some of the basic residues surrounding the cleft. Of the basic residues lining the substrate binding cleft in heparanase, only Arg^{272} and Arg^{303} are strictly conserved in Hpa2; Arg^{273} is replaced with lysine and His^{230}-Lys^{231} becomes a lysine and a serine, but Lys^{158}, Lys^{159}, and Lys^{161} are not conserved. Similarly, a number of the residues involved in hydrogen bonding with the HS substrate in heparanase are also not conserved. Collectively these findings suggest that if this cleft is available for HS binding, the affinity of the interaction would be lower than that seen with heparanase. However, as Hpa2 has a higher affinity for heparin and cell surface HS than heparanase (16), and given the likely position of the linker domain, the site(s) where heparin/HS binds is not the substrate binding cleft of heparanase. A mAb targeting the heparin/HS binding site on Hpa2 has been reported based on the fact that it inhibits Hpa2 binding to cell surfaces (83). As Hpa2 co-localizes with syndecan-1 on cell surfaces and cell surface binding is inhibited by heparin (16), this is a reasonable conclusion. However, the epitope on Hpa2 recognized by this mAb is not known. The structural similarity of the HS binding site on Hpa2 to the HS binding site on proheparanase, used for cell internalization via cell surface syndecans, is also unknown. To obtain a better understanding of this issue, we subjected both proheparanase and Hpa2 (modeled as shown in **Figure 3**) to an analysis of HS binding sites using the ClusPro server. This revealed that the most favored region for binding heparin tetrasaccharides is the C-domain of Hpa2, in contrast, for proheparanase it is the region around the heparin binding domain-2 (HBD2) (**Figure 4**). Thus, it is likely that Hpa2 binds to cell surface HS via its C-domain. An examination of the sequence alignment of these two proteins indicates that Hpa2 has what appears to be an extended heparin binding region in its C-domain spanning amino acids Lys^{449} to Arg^{480}. This covers the third potential HS binding region in heparanase, Lys^{411}-Arg^{432}, and includes the motif KRRKLR (48), which in heparanase, is not involved in HS binding as it is largely buried. In Hpa2 the motif region is: ^{465}QR*KPRPGRVIRD*KLR^{479} (the inserted sequence present only in Hpa2 is in italics) and although much of it is buried Arg^{466} is exposed. The analysis shown in **Figure 4** indicates that Arg^{466} acts in conjunction with the basic residues in the Hpa2 sequences, His^{505}-Lys^{512} and Arg^{561}-Thr^{568}, to form the HS binding region (**Figure 4**). Thus, the residues indicated by the ClusPro docking analysis to be involved in binding heparin tetrasaccharides are: Gln^{524}, Arg^{466}, Lys^{509}, Arg^{508}, Lys^{510}, Lys^{512}, Arg^{561}, Arg^{564}, Arg^{567}, and Thr^{568}. From

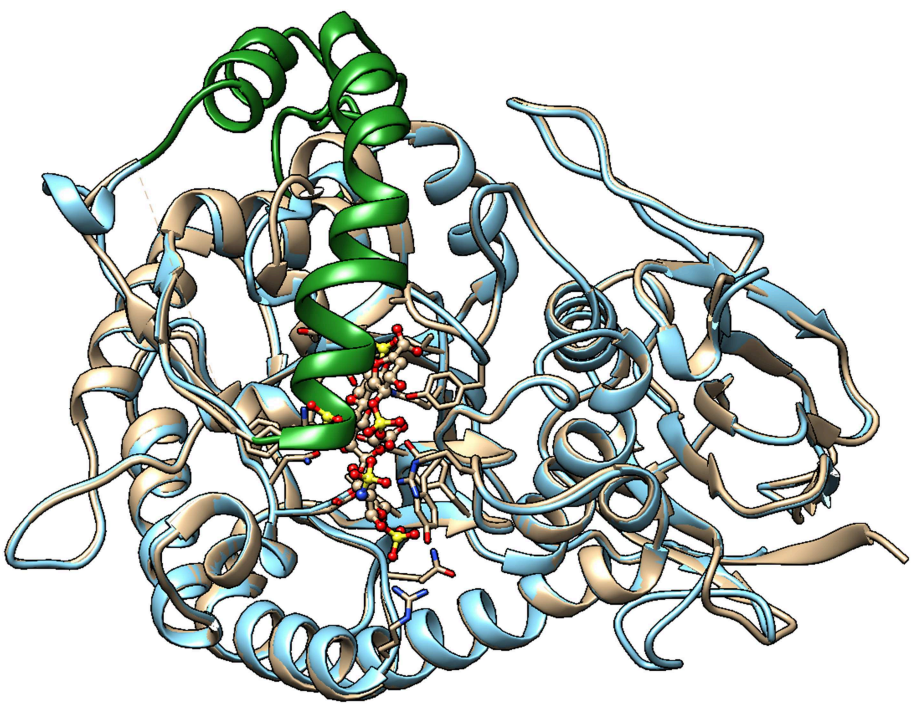

FIGURE 3 | Homology model of Hpa2 was built using the SWISS-MODEL server. PDB 5LA4 (Proheparanase) was used as a template. The obtained homology model of Hpa2 was then superimposed onto active heparanase (PDB code: 5e9c) cocrystal with heparin tetrasaccharide substrate. The linker (green ribbon) of Hpa2 partially occupies the heparin binding site. This is also true for proheparanase (PDB code:5LA4 structure). Heparanase is shown in golden, and Hpa2 in blue.

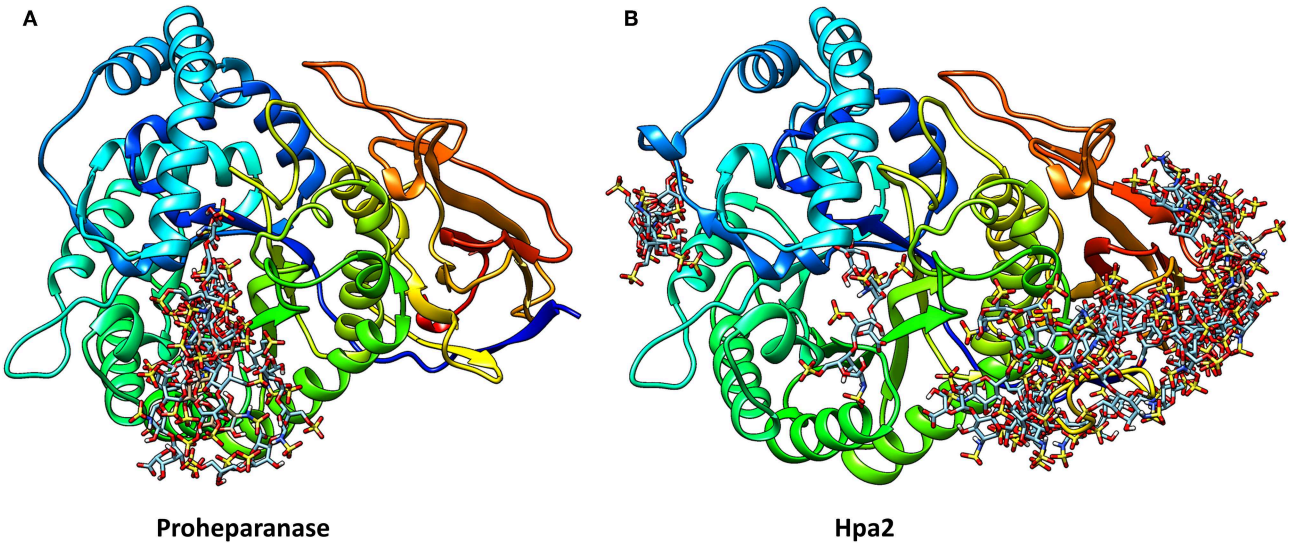

FIGURE 4 | The structures of proheparanase **(A)** and Hpa2 **(B)** are shown in ribbon representation and colored from N- (blue) to C- terminal (red). Prediction of heparin tetrasaccharide binding site using the ClusPro server suggests that most of the tetrasaccharides interact with residues 270–280 in proheparanase, whereas most of the interactions with the tetrasaccharides are concentrated toward the C-terminal domain of Hpa2.

the sequence alignment of heparanase and Hpa2, it is apparent that heparanase lacks three of these basic residues, three are conserved and for two residues the charge is retained, but arginine is replaced with a lysine and vice versa. These differences appear to be sufficient to stop heparin from binding to this region in heparanase (**Figure 4**).

The high affinity of Hpa2 for HS on cell surfaces has direct consequences for heparanase processing and activity. Hpa2 inhibits the internalization of heparanase and hence the processing and activation of this enzyme. It also markedly inhibits the enzymatic activity of purified, cell-free, active heparanase (16). Both of these effects can be attributed to competition for HS binding. However, co-immunoprecipitation data indicated that heparanase and Hpa2 could physically associate (16) and given a physical association is possible, there are two additional ways the enzymatic activity of heparanase may be inhibited by Hpa2. Firstly, the direct binding of Hpa2 to active heparanase may block enzymatic activity, and secondly, because Hpa2 is retained on cell surfaces, it may bind and retain latent heparanase in this location thereby preventing heparanase internalization and activation. It is also possible that the physical association of Hpa2 with heparanase may alter some of the non-enzymatic functions of heparanase. Curiously, Hpa2 expression does not always inhibit heparanase activity, as heparanase activity remained unchanged in pharyngeal carcinoma cells and in bladder carcinoma cells engineered to overexpress Hpa2 (83, 84).

Hpa2 and Cancer Biology

The expression of Hpa2 was first reported to be upregulated in colorectal cancer compared to normal tissues (85) and there are a number of studies where the expression of Hpa2 was found to be inversely correlated with disease severity. This was initially reported for head and neck carcinoma where there was an inverse correlation between Hpa2 staining intensity and tumor cell dissemination to lymph nodes, and an association between Hap2 expression and prolonged follow-up or disease recurrence (16). Similarly, in bladder carcinoma, an inverse correlation between Hpa2 staining and tumor grade was reported: weak or no Hpa2 staining was found in stage III tumors, whereas stage I tumors stained strongly (84). Whilst normal tissue adjacent to the head and neck carcinoma lesions did not stain for Hpa2, this was not the case for normal bladder epithelium, which stained strongly (16, 84). In the majority (68%) of melanoma metastasis samples, Hpa2 staining was detected, but this dropped to very low levels for brain metastases (86). Xenograft studies using pharyngeal carcinoma cells and bladder carcinoma cells overexpressing Hpa2 revealed Hpa2 expression attenuated tumor growth and was associated with the appearance of differentiation markers, increased collagen deposition and the induction of lysyl oxidase (83, 84). Reduced cell proliferation and reduced blood vessel densities were also detected in the pharyngeal carcinoma model as was a reduction in the expression of Id1, a proangiogenic transcription factor, which induces the expression of VEGF isoforms (83). Similar data were obtained in a pancreatic carcinoma model leading to the interpretation that Hpa2 functions as a tumor suppressor in these carcinomas in a heparanase and HS independent manner (17).

HEPARANASE AS A DRUG TARGET

Criteria for a Good Target

It is clear that heparanase has a profound role in the pathophysiology of a variety of different types of cancers; its increased expression is associated with greater tumor size, more angiogenesis, greater metastatic tendencies, and poor prognosis. In addition, as its genetic knockdown in experimental disease

models significantly curtails tumor progression (41, 42), it can be concluded that heparanase is a valid cancer drug target. But is it a challenging drug target?

Frequently successful therapeutic targets are enzymes (87), and heparanase is an enzyme. Its substrate specificity is restricted to particular HS or heparin structures, and as revealed by its crystal structure, its active site is a cleft that has strict structural requirements for access and stable binding (25). From a structure-based drug discovery viewpoint this is a good thing. Importantly, crystal structures are now available for both the active enzyme and its latent precursor (25, 30). This means that it is possible to accurately predict where on heparanase a potential drug is binding. This information can then be used to inform the medicinal chemistry, thereby leading to the rational design of compounds that better modulate heparanase activity. For example, the crystal structure could be used to design a compound that fits into the catalytic cleft and so specifically blocks enzymatic activity. A further criterion for a good drug target is its "assayability" (87). For heparanase, the development of good, reliable assays to monitor its enzymatic activity in a manner that is appropriate for high throughput screening has been problematical, with many of the assays being complex and labor intensive. Nevertheless, the development of simple, synthetic substrates with a single point of cleavage that will allow detailed kinetic analyses to be performed may well-lead to the standardization of heparanase assays (88).

Good drug targets are generally not expressed uniformly throughout the body, and their modulation in normal, non-disease situations should be markedly less critical than in the disease (87). Such is the concentration of recent literature on the contribution of heparanase to a variety of different diseases that the reader would be forgiven for concluding that heparanase contributes minimally, if at all, to normal physiological processes. In normal tissues, heparanase expression is restricted to the placenta, lymphoid organs, leukocytes, platelets, keratinocytes, and endothelial cells and mice deficient in heparanase expression lack gross anatomical abnormalities (18). However, heparanase knock-out mice are not without a phenotype. For example, heparanase has a profound role in wound healing. It is normally found in skin and wound granulation tissue, where it stimulates keratinocyte migration and epithelialisation during healing, as well as angiogenesis and blood vessel maturation (89). Curiously heparanase was found to regulate hair growth, hair homeostasis and the differentiation of the inner root sheath in hair follicles (90). In the bone marrow, heparanase modification of the microenvironment was shown to regulate the retention and proliferation of progenitor cells (91). Bone marrow mesenchymal stem cells are weak expressers of heparanase and loss of heparanase activity reduced their self-renewal and proliferation (92). Collectively these data suggest that heparanase has a role in normal stem cell biology. Heparanase also has a role in normal developmental processes like mammary gland development, where its enzymatic activity is required for mammary epithelial invasion and branching (93). Leukocyte heparanase is necessary for immune cell migration into tissues. This was found to be the case for dendritic cells, NK cells, monocytes and macrophages (18, 53, 54, 94, 95). Moreover, heparanase expression is required for the activation of macrophages, and in mast cells, heparanase is an important regulator of protease storage in mast cell granules (53, 96). Given these findings neutralizing heparanase systemically may alter a patient's ability to mount an efficient immune response to an infection, or to a tumor, and to heal wounds in a timely manner. However, it is recognized that knocking out a gene and neutralizing activity via administering a drug are quite different things, as in the latter, drug penetrance will not be 100% and this may vary according to the route of administration. Given that minor side-effects can be tolerated in an anti-cancer drug, it is fair to say that heparanase scores quite positively on this point.

CHALLENGES FOR HEPARANASE AS A DRUG TARGET

Heparanase is a multi-functional protein with different parts of the protein being involved in differing aspects of its biological activity. It should be clear from the above discussion that both the enzymatic and non-enzymatic activities of heparanase are likely to contribute to the role this protein plays in cancer pathology. Although the catalytic cleft is involved in the enzymatic activity, it is not clear which regions of heparanase are involved in its non-enzymatic activity. The fact that the T5 splice variant of heparanase, which lacks enzymatic activity and heparin binding capability, also enhances tumor growth and is associated with a poor prognosis raises the question as to the relevance of the enzymatic activity of heparanase in cancer biology. This question is not clarified by the genetic heparanase silencing experiments because this methodology would knock-down both T5 and full-length heparanase. Given the data on the T5 variant, the question must be asked as to whether the enzymatic activity is the critical function of this protein that causes its pro-tumor and pro-metastasis effects.

It could be argued that the good pre-clinical data of the various heparanase inhibitors, selected based on their ability to block enzymatic activity, indicates the importance of HS degradation in tumor biology. However, as the best-studied inhibitors have pleiotropic effects that are not confined to inhibiting the enzymatic activity of heparanase, this argument may not be valid. For example, PG545 was selected from a series of similar structures because of its ability to bind the angiogenic growth factors FGF1, FGF2, and VEGF (presumably via its sulfated saccharide moiety) as well as for its inhibition of the enzymatic activity of heparanase (97, 98). Moreover, recent studies have indicated that the mode of action of PG545 is likely to be complex. Curiously PG545 has been found to elicit autophagy and persistent ER stress in lymphoma cells, which triggered their apoptosis, and this occurred independently of heparanase (99). Whereas, in glioma, PG545 attenuated heparanase augmented autophagy, and this reduced autophagy was associated with decreased cell growth and decreased tumor load (64). PG545 has also been reported to have immunomodulatory effects that are distinct from heparanase, and its stimulation of dendritic cells to produce IL-12 via a TLR9-dependent pathway which then activates NK cells, is now regarded as a key part of

the anti-tumor effect of PG545 (100, 101). Similarly, M-402 was designed to inhibit VEGF, FGF2, stromal cell-derived factor (SDF)-1α, P-selectin as well as heparanase (102), and SST0001 inhibits the activation of a number of receptor tyrosine kinases, in addition to its effects on heparanase (103). A recent publication confirmed M-402 was a broad, multi-targeting drug *in vivo* and one of its very interesting effects relevant to pancreatic cancer invasion/metastasis was on the levels of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-3 (TIMP3); it reduced the former and increased the latter (104). Thus, using one or more, of these well-studied inhibitors to confirm that the biological activity under study is due to the enzymatic activity of heparanase is problematical.

To be more certain of targeting heparanase's enzymatic activity has required the development of blocking anti-heparanase mAbs (105). Two mAbs that block enzymatic activity have been reported. One mAb (9E8) was raised using the heparanase peptide Lys^{158}-Asp^{171} as the antigen, whilst the other (H1023) used intact active heparanase as the antigen and the hybridomas produced were screened for heparanase binding and inhibition of enzymatic activity (105). Both mAbs inhibited lymphoma growth in xenogeneic murine models via a mechanism that did not involve a direct cytotoxic effect on the tumor cells, but rather seemed to be a result of neutralization of heparanase activity in the tumor microenvironment. Interestingly, these mAbs also inhibited spontaneous metastasis arising from murine ESb T-lymphoma cells. Tumor growth and angiogenesis were further constrained when both mAbs were administered together implying that greater efficacy is achieved when two different epitopes on heparanase are targeted (105). The peptide antigen is equivalent to HBD1. Given that Lys^{158} and Lys^{159} are available for HS binding in proheparanase, and that the mAb 9E8 inhibited proheparanase uptake by cells as well as heparanase enzymatic activity, it is probable the epitope recognized by this antibody is at the extreme N-terminus of this peptide. Although a three dimensional epitope in the region of the catalytic cleft appears likely for H1023, it is still conceivable that H1023 binding to a site on the β-sandwich C-domain may allosterically influence access to the catalytic cleft. Regardless, these mAbs are recognizing different epitopes. Given these data, it appears at first glance that inhibition of proheparanase uptake and heparanase enzymatic activity by mAbs is sufficient for significant anti-lymphoma activity. However, the extent to which non-enzymatic activities attributed to heparanase are also inhibited by these mAbs is not known. In this context it is important to recognize that an intact antibody molecule is approximate twice the mass of heparanase, thus although the heparanase epitope recognized may comprise a few amino acids the area on heparanase masked by antibody binding will be much greater. Interestingly, the efficacy of PG545 in inhibiting tumor growth appears to be superior to these mAbs on weight of drug administered basis, in the dosing regimen used (105). Although, how much of the activity of PG545 is due to its interactions with heparanase and how much to its other activities is impossible to say.

The fact that heparanase is such a multi-functional protein makes it a challenging drug target. All of the heparanase inhibitors under development include in their biological assay portfolio an assay for this protein's enzymatic activity, but other aspects of heparanase's activities that cannot be attributed to its enzymatic activity are generally not well-studied. Nor is it entirely clear which regions of the protein are involved in these non-enzymatic activities.

Evidence has been presented to suggest that the COOH-terminal domain (C-domain) mediates some of the non-enzymatic functions of heparanase, including Akt phosphorylation and cell proliferation, and it facilitates tumor progression in a glioma xenograft model (26). The C-domain, comprising amino acids 413–543, forms a β-sandwich domain comprising eight β-strands arranged in two sheets, with the 8 kDa N-terminal fragment contributing one of the β-strands (25, 26). This domain structure is stabilized by both hydrophobic interactions and a disulfide bond linking Cys^{437} to Cys^{542} (28) (**Figure 1**). Although not directly involved in the formation of the catalytic cleft, the C-domain is required for enzymatically active heparanase and heparanase secretion. Deletion of the COOH-terminal amino acids Phe^{527}-Ile^{543} was found to cause loss of enzymatic activity (26), and more particularly, mutation of Leu^{522}, Leu^{524}, Phe^{531}, Phe^{532}, Val^{533}, or Ile^{534} within a conserved hydrophobic region in the C-domain also abolished activation of heparanase (27).

Much of the initial work examining functional activities of the C-domain used a construct that lacked the β-strand contributed by Gln^{36}-Ser^{55} from the 8 kDa fragment. Although when expressed, this C-domain localized to the Golgi apparatus and stained with a polyclonal anti-heparanase antibody, both features suggestive of a correctly folded protein, its secretion was modest (26). This raised a query as to the stability of this domain in the absence of the β-strand from the 8 kDa *N*-terminal fragment. Accordingly, the expression of a construct comprising Gln^{36}-Ser^{55} linked to the C-domain sequence was examined. This protein, termed 8C, was secreted at levels comparable to native heparanase, it markedly induced Akt phosphorylation, and triggered endothelial cells to form tube-like structures in accordance with the proangiogenic activity of intact heparanase. Unfortunately, glioma progression was not studied with the 8C protein (26). When the regulatory elements of the mouse mammary tumor virus (MMTV-LTR) were used to direct expression of the 8C protein to the mammary epithelium of mice, over time (6–10 months) these animals (MMTV-8C mice) spontaneously developed tumors in their mammary and salivary glands (106). These invasive carcinomas were highly proliferative and phosphorylated signaling proteins, STAT3 (signal transducer and activator of transcription), Erk and Akt, were detected in the tumor tissue. In contrast, heparanase transgenic mice did not spontaneously develop tumors. Curiously, the signaling molecules that were phosphorylated in non-transformed cells were not identical for MMTV-8C and MMTV-heparanase mice (mice in which wild-type heparanase expression is regulated by MMTV-LTR) (106). Clearly more work is required to determine whether the 8C protein or the C-domain as expressed in these studies are good models for understanding the function of the native C-domain in wild-type heparanase.

A further complication for heparanase as a drug target is that it is present in extracellular locations and intracellularly, including within the nucleus. The nature of many heparanase inhibitors, that is their size and charge, is such that they cannot gain access into the cell unless they are transported by ligands into the endosomal pathway. The heparin mimetic-type heparanase inhibitors currently in clinical trial will inhibit heparanase internalization mediated by cell surface HS-proteoglycans, and as the internalization of latent heparanase is required for processing it may be thought that these inhibitors will also halt processing. However, these drugs are unlikely to inhibit heparanase uptake that is independent of cell surface HS. Importantly, heparanase binding with high affinity to cation independent mannose 6-phosphate receptors [CIMPR, also called CD222 and insulin-like growth factor 2 receptor (IGF2R)] leads to its internalization in a way that is independent of HS and is not inhibited by heparin (107). Given that CIMPR is ubiquitously expressed and functions in the recycling of growth factors and other ligands it is probable that significant quantities of heparanase are internalized by this mechanism, and particularly so in cells like leukocytes or lymphoma cells that have relatively low levels of surface HS. In the presence of anti-heparanase drugs, this non-HS dependent mechanism is likely to dominate. It could be argued that heparanase located in endosomal pathways is "protected" from the action of the anti-heparanase drugs currently in a clinical trial. This argument also holds for heparanase in the nucleus. Hence, even in the presence of the drug, nuclear heparanase would continue to protect cancer cells from apoptosis by its ability to down-regulate the transcription of pro-apoptotic genes (77), a function that does not involve enzymatic activity.

Despite the structural similarities between heparanase and Hpa2, very little attention has been paid to the latter protein in relation to whether or not it binds to, or sequesters, anti-heparanase drugs, and so down-modulates their activity. Although in many instances, Hpa2 expression is inversely correlated with cancer progression, this is not always the case. For example, in differentiated thyroid carcinoma, both heparanase and Hpa2 are over expressed relative to benign lesions, but Hpa2 expression is extremely elevated, and this expression is confined to neoplastic cells (108). Similarly, in squamous cervical cancer, a progressive increase in Hpa2 expression according to the severity of the lesion was recorded (109). It is known that Hpa2 can down-modulate heparanase processing and enzymatic activity by competing for HS, but does this remain the case in the presence of anti-heparanase heparin mimetic drugs? To our knowledge, this question has not been examined. Given the structures of the Glycol-split heparin mimetic drugs, SST0001 and M-402, it is very likely these drugs would bind Hpa2 at least as well as, if not better than, they bind heparanase. If binding does occur this could decrease the ability of Hpa2 to inhibit heparanase activity.

STRUCTURAL ASPECTS OF HEPARANASE INHIBITORS

Prior to publication of the crystal structure of active heparanase in 2015 the majority of non-antibody heparanase inhibitors were either modified natural heparin molecules (e.g., M-402 and SST0001), sulfated oligosaccharides derived from marine organisms (e.g., carrageenans), semisynthetic compounds comprising oligosaccharide backbones that were chemically sulfated (e.g., PI-88, JG3, and PS3), or as is the case for PG545 a sulfated saccharide component covalently functionalised with cholestanyl aglycone (20, 110). The goal was to mimic heparin's structure but remove its anticoagulant activity. Although heparanase can cleave heparin, the majority of the heparin chains released from mast cells lack the cleavage site and therefore act as non-cleavable inhibitors. The heparanase crystal structure indicated that these heparin mimicking inhibitors would not bind in the catalytic cleft, but rather were binding to the heparanase surface in a manner that masked the cleft thereby preventing access to HS chains with cleavage sites. Studies of PG545 binding kinetics provided clues as to its mechanism of heparanase inhibition. It was found to bind heparanase with parabolic kinetics indicating that two sites on heparanase are involved in PG545's binding and inhibition (111). The identification by homology modeling of a hydrophobic channel on heparanase surrounding the catalytic resides (33) suggested a mechanism. It was proposed that the sulfated saccharide moiety in PG545 binds to the positively charged amino acids of the HS binding regions whilst the cholestanol aglycone component occupies the hydrophobic channel (or cleft) thereby obscuring the catalytic site. It was further proposed that this cholestanol, stabilized on heparanase through its binding in the hydrophobic channel, then serves as a binding site for the cholestanol group of another PG545 molecule (111). The result being that PG545 is a more potent inhibitor of heparanase enzymatic activity than similar compounds that lack the cholestanol aglycone (97, 111).

Recently dendrimer glycomimetics that are heparanase enzymatic activity inhibitors were developed (112). The rationale being that the avidity of weak interactions, of the type mediated by sugar fragments binding a protein, is significantly enhanced if multiple copies of the sugar units are displayed on a chemically defined scaffold. The most potent of these dendrimer glycomimetics, with a potency almost comparable to PG545, comprised individual sulfated maltose units linked to form a tetrameric cluster, such that the terminal groups of the dendrimer arms were the sulfated disaccharides. Given the similarity in the basic structure of the saccharide components of PG545 and the dendrimer, it is possible that the latter compound also binds to the positively charged amino acids surrounding the catalytic cleft, rather than in the cleft itself. However, no modeling or structural data were provided to verify this suggestion. This compound was shown to inhibit tumor growth and metastasis in a mouse xenograft model with human myeloma cells (112), but no other activities described for heparanase were examined. Glycopolymers that inhibit heparanase enzymatic activity have also been prepared. A set of glycopolymers comprised a poly-2-aminoethyl methacrylate (PAMA) backbone to which heparin disaccharides, derived by extensive digestion of heparin with heparinase I, II and III, were covalently coupled, and then any unreacted amines were sulfated (113). These compounds were heterogeneous as specific disaccharides structures were not selected prior to coupling. As well as inhibiting heparanase

enzymatic activity a selected glycopolymer was further tested in *in vitro* invasion and migration assays using the murine B16 melanoma line; activities which may be attributed to heparanase. The dendrimers and the glycopolymers were prepared without reference to the structure of the catalytic cleft, or to the specificity of the bond that is cleaved in HS, rather they were primarily designed as heparin mimetics.

In contrast, others have used knowledge of the HS bond that is cleaved by heparanase to inform their choice of the disaccharide unit used as the active entity in their anti-heparanase glycopolymers. In particular, as GlcAβ(1,4)GlcNS(6S) is cleavable, GlcNS(6S)α(1,4)GlcA was chosen because it is not cleaved, yet is a structure that fits and binds within the catalytic cleft of heparanase (114, 115). Thus, a glycopolymer, demonstrated to possess comparable anti-metastatic activity to SST0001, and comparable inhibition of heparanase enzymatic activity as heparin at 10 μg/ml, comprised 12 repeating units of pendent GlcNS(6S)α(1,4)GlcA saccharides (115). However, the inhibition of other heparanase activities that do not require enzymatic activity was not addressed. This glycopolymer lacked anticoagulant activity and bound poorly to the angiogenic growth factors, FGF1, FGF2, and VEGF. It also bound poorly to platelet factor 4 (PF4), suggesting it may not trigger thrombocytopenia as was the case with PI-88 (110).

The publication of the crystal structure of heparanase has allowed the development of small molecule drugs, the designs of which were aided by molecular modeling to inform of their likelihood of binding within the catalytic cleft. Appropriately designed small molecule drugs have the potential to exploit the characteristics of the catalytic cleft to achieve high binding affinities and favorable pharmacokinetic properties, and they may also be orally available. To date, none of these compounds have entered a clinical trial. A number of these small molecule, synthetic anti-heparanase inhibitors have been discussed in a recent publication (20) and will not be further reviewed here.

Nevertheless, of particular interest are the benzazole derivatives that have been developed using medicinal chemistry and molecular docking into the catalytic cleft to design compounds with a good fit and nanomolar anti-heparanase enzymatic activities. Two sets of derivatives have been synthesized, asymmetrical, and symmetrical derivatives (116, 117). The most recent symmetrical compounds have a relatively rigid central portion, sufficient length to span the catalytic cleft, and terminal acidic groups. Collectively these characteristics allow the compounds to fit into the cleft quite well. They occupy the same binding position as the HS tetrasaccharide that was co-crystallized with heparanase. Thus, the amino acids that interact with the HS substrate also interact with the synthetic inhibitors and the flexible, terminal acidic groups interact with the polar and basic amino acids at either end of the cleft. The best anti-heparanase of these, in terms of inhibiting heparanase enzymatic activity, is a symmetrical, thiourea glycine benzoxazole compound having an IC_{50} of 0.08 μM (117). As this compound has yet to be tested in animal models, it is impossible to say whether or not it is a good anti-cancer agent. Nevertheless, it has been shown to inhibit the invasion of a number of different tumor cell lines in an *in vitro* Matrigel assay, at concentrations that do not affect cell proliferation. Most interestingly it also inhibited the transcription and mRNA levels of the proangiogenic proteins, FGF1, FGF2, VEGF, and the metalloproteinase MMP-9 as well as heparanase itself (117). However, whether this effect was dependent upon heparanase was not determined.

CONCLUSIONS

It is apparent from the analysis described here that heparanase is not a straight-forward anti-cancer drug target despite the wealth of evidence to indicate it contributes to tumor growth, tumor cell migration, metastasis formation, and chemoresistance. It is difficult to know how many of its contributions to cancer biology are due to its enzymatic activities, its non-enzymatic activities, or its contributions in the nucleus to the pattern of genes that are transcribed. Given this, the question is which parts of the protein should be targeted in drug development? The crystal structure revealed the catalytic cleft of heparanase is well-suited to small molecule drug development and high affinity binding by compounds like the benzazole derivatives, but whether small molecule drugs that only bind within the enzymatic cleft will inhibit the plethora of heparanase's activities *in vivo* is doubtful. Nevertheless, the *in vivo* data obtained from testing these small molecule drugs in various tumor animal models should be quite informative, and particularly so if, in these studies, analyses are also performed to reveal the expression levels of activated heparanase, the T5 heparanase variant, and Hpa2 in the tumor and its surrounding microenvironment. It is possible the *in vivo* efficacy of the anti-heparanase drugs may vary according to the expression levels of these latter two proteins, even though it is unlikely that a small molecule drug designed to specifically bind in the catalytic site will also interact with the T5 variant or Hpa2. Data obtained from *in vivo* testing of catalytic cleft specific small molecule drugs may better reveal the relative importance of the enzymatic function of heparanase, compared to its non-enzymatic activities, in tumor progression, than was the case with the anti-heparanase drugs that have currently entered clinical trial.

Clearly the catalytic cleft should be targeted, but maybe the HBD2 around residues 270–280 should also receive attention. Targeting these two areas with a "hybrid" drug comprising a small molecule like component designed to fit into the catalytic cleft, plus possibly a negatively charged saccharide-like component which would bind HBD2 outside of the cleft, may produce a drug which inhibits heparanase's enzymatic activity as well as some of its non-enzymatic activities. Moreover, binding of the negatively charged component could guide the small molecule section into the more hydrophobic catalytic cleft and so increase the kinetics of binding. However, the structure of such a negatively charged component should be informed by molecular modeling to prevent it from also interacting with the C-domain of Hpa2.

Importantly, it has been demonstrated that *in vivo* the anti-heparanase drugs will act on heparanase secreted by both the

tumor and the host (20, 105), a fact that is often overlooked when assessing the *in vivo* data. Also overlooked is the fact that anti-heparanase drugs given systemically will act on the heparanase secreted by the host's tumor invading immune cells, and this could retard their immune-surveillance protective effects and so allow tumor growth. Certainly, active heparanase is of major importance for NK cell invasion of tumors and macrophage activation (53, 54).

The extent to which the issues raised in this manuscript have impeded anti-heparanase drugs from entering the clinic is unknown. Despite these issues, we believe heparanase remains a useful therapeutic target in the battle against cancer metastasis. It maybe that the clinical trials conducted to date have recruited patient populations that are too diverse in their disease, leading to an overall apparently poor response, although some patients did respond well to treatment. Whether this good response to therapy was because of the anti-heparanase activity of the drugs or their other activities is impossible to say.

AUTHOR CONTRIBUTIONS

DC was responsible for conceptualization, writing and project management. NG prepared the figures and provided insight into structural aspects discussed in work. DC and NG listed above have made an intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Regulation of Heparanase in Diabetes-Associated Pancreatic Carcinoma

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While at least six types of cancer have been associated with diabetes, pancreatic ductal adenocarcinoma (PDAC) and diabetes exhibit a unique bidirectional relationship. Recent reports indicate that majority of PDAC patients display hyperglycemia, and ∼50% have concurrent diabetes. In turn, hyperglycemic/diabetic state in PDAC patients fosters enhanced growth and dissemination of the tumor. Heparanase enzyme (the sole mammalian endoglycosidase degrading glycosaminoglycan heparan sulfate) is tightly implicated in PDAC progression, aggressiveness, and therapy resistance. Overexpression of heparanase is a characteristic feature of PDAC, correlating with poor prognosis. However, given the lack of heparanase expression in normal pancreatic tissue, the regulatory mechanisms responsible for induction of the enzyme in PDAC have remained largely unknown. Previously reported inducibility of heparanase gene by diabetic milieu components in several non-cancerous cell types prompted us to hypothesize that in the setting of diabetes-associated PDAC, hyperglycemic state may induce heparanase overexpression. Here, utilizing a mouse model of diet-induced metabolic syndrome/diabetes, we found accelerated PDAC progression in hyperglycemic mice, occurring along with induction of heparanase in PDAC. *In vitro*, we demonstrated that advanced glycation end-products (AGE), which are largely thought as oxidative derivatives resulting from chronic hyperglycemia, and the receptor for AGE (RAGE) are responsible for heparanase induction in PDAC cells. These findings underscore the new mechanism underlying preferential expression of heparanase in pancreatic cancer. Moreover, taken together with the well-established causal role of the enzyme in PDAC progression, our findings indicate that heparanase may sustain (at least in part) reciprocal causality between diabetes and pancreatic tumorigenesis.

Keywords: heparanase, pancreatic carcinoma, diabetes, hyperglycemia, extracellular matrix

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest forms of malignancy and expected to become the second-leading cause of cancer-related death in the United States by 2030 (1). Dysregulation of glucose metabolism occurs in majority of PDAC patients: at PDAC diagnosis up to 85% of subjects have hyperglycemia and ∼50% have diabetes (2–4). In a subset of PDAC patients diabetes occurs as early as 1–3 years before a detection of PDAC and is regarded as "new onset diabetes" (2–5). Long-standing type 2 diabetes also acts as a risk factor for pancreatic

cancer (6, 7). Thus, elevation of glucose is a common phenomenon in PDAC (2–4). Additionally, positive association was reported between PDAC and insulin resistance/hyperinsulinemia (3, 8, 9). Conversely, recent reports suggest that diabetic state promotes PDAC and renders it highly aggressive, resistant to the existing therapies, and is associated with extremely poor prognosis (3, 4, 10–12). Hence, PDAC and diabetes exhibit a unique bidirectional relationship, with diabetes being both an effect and etiological factor of the pancreatic cancer (3, 4, 11).

Overexpression of heparanase (the only known mammalian endoglycosidase capable of degrading glycosaminoglycan heparan sulfate [HS]) is a characteristic feature of PDAC and correlates with its agressiveness/poor prognosis (13–17). HS proteoglycans are ubiquitously found both at the cell surface and in the extracellular matrix (ECM) (18–20). HS chains bind to and assemble ECM proteins, thus playing important roles in ECM integrity and cell-ECM interactions (18–20). In addition, HS chains regulate the activity of a variety of bioactive molecules (i.e., cytokines, growth factors) at the cell surface and in the ECM (21–24). The link between heparanase and PDAC progression is well-established (14–17) and the underlying molecular/cellular mechanisms include increased invasiveness (13, 25) and creation of tumor-promoting inflammatory environment (26). However, given the lack of heparanase expression in normal pancreatic tissue (13, 27), the regulatory mechanism(s) responsible for induction of the enzyme in PDAC are largely unknown.

Notably, heparanase was implicated in diabetes and its complications (17, 28–32). Moreover, previous research revealed molecular mechanism responsible for heparanase induction in immune, endothelial, and epithelial cells by several diabetic milieu components, i.e., high glucose, advanced glycation end-products, free fatty acids (29–31, 33–36). These findings, along with the impaired glucose metabolism that typically occurs in PDAC patients (3, 4), prompted us to hypothesize that in the setting of pancreatic carcinoma and associated hyperglycemia, constituent(s) of the diabetic milieu could be responsible for heparanase induction in PDAC cells.

Here, applying *in vivo* model of diet-induced metabolic syndrome [a cluster of conditions that includes hyperglycemia, insulin resistance, hyperinsulinemia, diabetes, and obesity (37)], we found that accelerated PDAC progression in mice with impaired glucose metabolism coincided with induction of heparanase in pancreatic tumors. *In vitro*, we demonstrated that advanced glycation end-products [AGE, oxidative derivatives resulting from hyperglycemia, whose levels are increased in clinical/experimental diabetes (38–42)] and its receptor (RAGE) are responsible for upregulation of heparanase in PDAC cells. AGEs form at a constant but slow rate in the normal body, however, their formation is markedly accelerated in diabetes because of the increased availability of glucose. Given deterioration in glycemic control in a majority of PDAC patients (2–4), these findings provide molecular explanation for induction of heparanase in pancreatic carcinoma. Moreover, taken together with the previously demonstrated causal role of the enzyme in PDAC progression (13, 26), our observations indicate that heparanase may be a part of the bi-directional link between diabetes and pancreatic tumorigenesis.

MATERIALS AND METHODS

Cell Culture

The mouse pancreatic carcinoma cell line Panc02 [(43), a generous gift from M. Dauer (Munich, Germany)], and human pancreatic carcinoma cell lines MIA PaCa2 and PANC-1 (authenticated by STR profiling at the Genomics Center of the Biomedical Core Facility, Technion University, Israel), was grown in DMEM supplemented with 1 mM glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% FCS (Biological Industries) at 37°C and 8% CO_2. At 60–80% confluence, cells were maintained for 24 h in serum-free DMEM, and either remained untreated or were incubated with AGE (AGE-BSA, catalog #JM-2221–10; MBL International Corporation), or BSA (Sigma-Aldrich). In some experiments Panc02 cells were pretreated with RAGE neutralizing antibody (AF1179, R&D Systems) or TAK-242, TLR4 inhibitor (InvivoGen). The final endotoxin levels in experimental media containing AGE/BSA were 0.024–8 pg/mL, which were significantly lower than the concentrations typically found in diabetic patients (44), or than those required to activate Toll-like receptor (TLR) 4 or the classic NFκB pathway (45, 46). Cells were lysed and processed for RNA isolation. In some experiments, cells were cultured on glass coverslips (12 mm; Carolina Biological Supply Company), fixed with 100% ice-cold methanol and processed for imunofluorescent staining.

Mouse Model of Metabolic Syndrome and Concurrent Pancreatic Carcinoma

Nine week-old male C57BL/6J mice ($n = 10$ per experimental group) were fed for 14 consecutive weeks with either regular (control) diet [Teklad 2018S] or the diabetogenic high fat diet (Teklad TD.06414), as in Montgomery et al. (47), Pettersson et al. (48), and Sandu et al. (49). At week 12, when experimental mice developed metabolic syndrome and became hyperglycemic, Panc02 pancreatic carcinoma cells were injected subcutaneously (10^6 cells per mouse). Volume of tumors was monitored for 2 weeks following injection, then animals were sacrificed and tumors were snap-frozen for protein extraction. Part of the tumor tissue was processed for histology. Mice were kept under pathogen-free conditions; all experiments were performed in accordance with the Hebrew University Institutional Animal Care and Use Committee.

Antibodies

Immunoblot analysis and immunostaining were carried out with the following antibodies: anti-phospho-AKT Ser 473 (Cell Signaling), anti-phospho NFκB p65 Ser276 (Cell Signaling Technology); anti-actin (Abcam); and anti-heparanase monoclonal antibody 01385–126, recognizing both the 50-kDa

subunit and the 65-kDa proheparanase (50), which was provided by Dr. P. Kussie (ImClone Systems).

Immunoblotting

Tumor tissue samples were homogenized in lysis buffer containing 0.6 % SDS, 10 mM Tris-HCl, pH 7.5, supplemented with a mixture of protease inhibitors (Roche), and phosphatase inhibitors (Thermo Scientific). Equal protein aliquots were subjected to SDS-PAGE (10% acrylamide) under reducing conditions, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 3% BSA for 1 h at room temperature and probed with the appropriate antibody, followed by horseradish peroxidase–conjugated secondary antibody (KPL) and a chemiluminescent substrate (Biological Industries). Band intensity was quantified by densitometry analysis using Scion Image software.

Immunohistochemistry

Paraffin-embedded slides were deparaffinized and incubated in 3% H_2O_2. Antigen unmasking was carried out by heating (20 min) in a microwave oven in 10 mmol/L Tris buffer

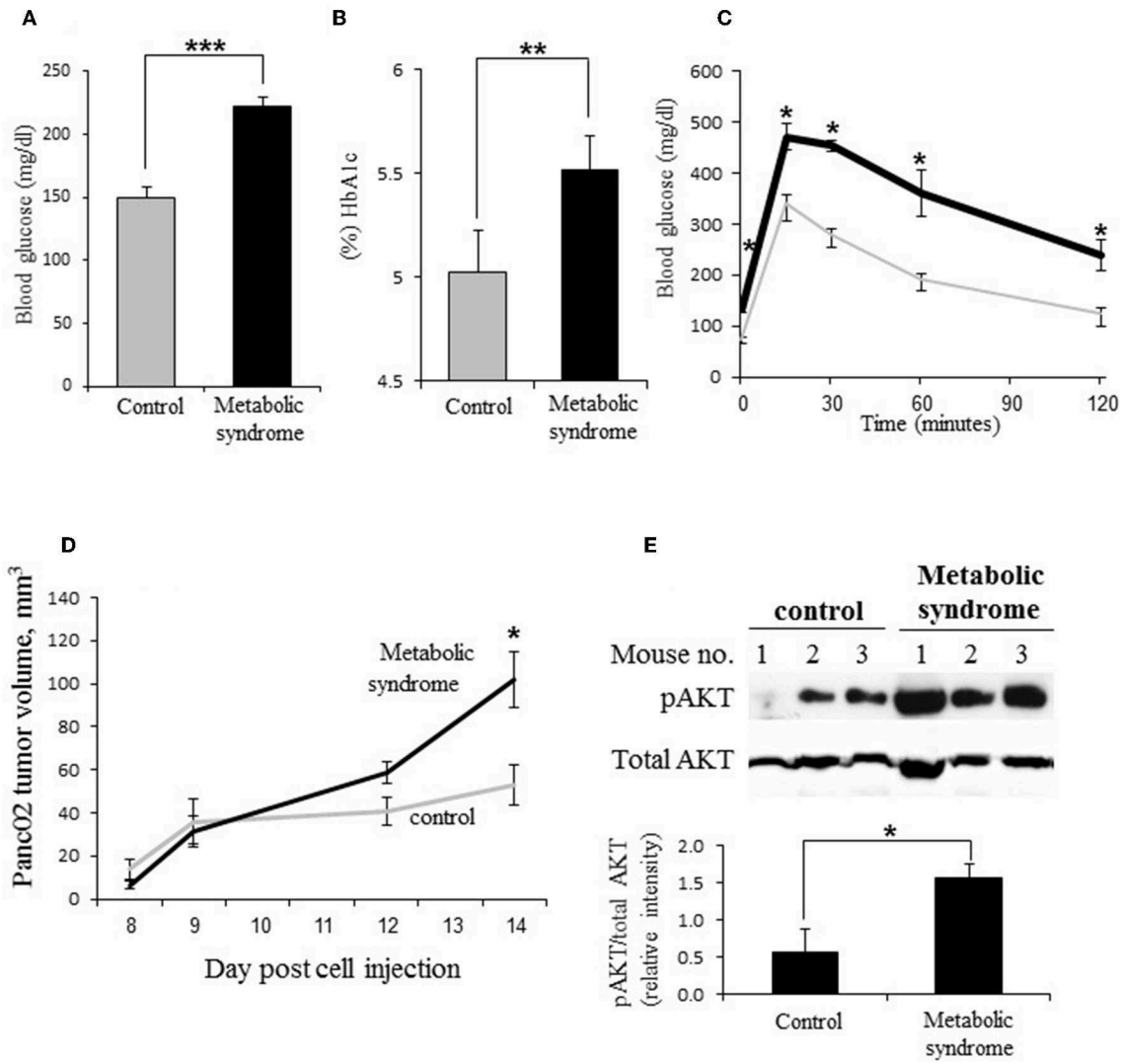

FIGURE 1 | Dysregulation of glucose metabolism accelerates PDAC progression *in vivo*. **(A–C)** Impairment of glucose metabolism in male C57BL/6J mice with diet-induced metabolic syndrome. **(A)** Blood glucose levels [mg/dL], **(B)** glycated hemoglobin [HbA1c, %], and **(C)** glucose tolerance (determined by the i.p. glucose tolerance test [GTT]) in male C57/Bl6 mice following 12 weeks of diabetogenic high fat diet (**A,B**: black bars; **C**: black line) or regular (control) diet (**A,B**: gray bars; **C**: gray line). Error bars represent ±SD. Two-sided Student's *t*-test $^{*}p \leq 0.003$, $^{**}p = 0.013$, $^{***}p = 0.0002$; $n \geq 5$ mice per condition. **(D,E)** On week 12, mouse pancreatic carcinoma Panc02 cells were injected subcutaneously (10^6 cells per mouse). **(D)** Volume of Panc02 tumors grown in mice with diet-induced metabolic syndrome (black line) and control mice (gray line) was monitored for 14 days. Error bars represent ±SE. Two-sided Student's *t*-test $^{*}p < 0.02$. **(E)** Top: Increased levels of phospho-AKT (pAKT) in Panc02 tumors of mice with metabolic syndrome as compared to control mice. Bottom: The band intensity was quantified using ImageJ software; intensity ratio for pAKT/total AKT is shown, error bars represent ±SE. Two-sided Student's *t*-test $^{*}p = 0.048$; $n \geq 3$ mice per condition.

containing 1 mmol/L EDTA. Slides were incubated with primary antibodies diluted in CAS-Block (Invitrogen) or with CAS-Block alone, as a control. Appropriate secondary antibodies (Nichirei) were then added, and slides were incubated at room temperature for 30 min. Mouse stain kit (Nichirei) was used when primary mouse antibodies were applied to stain mouse tissues. Color was developed using the DAB Substrate Kit (Thermo Scientific) or Zymed AEC Substrate Kit (Zymed Laboratories), followed by counterstaining with Mayer's Hematoxylin. Controls without addition of primary antibody showed low or no background staining in all cases. Immunohistochemistry was scored based on staining intensity, as described in figure legends.

Immunofluorescence

For immunofluorescence analysis, DyLight 549 donkey anti-mouse and Cy^{TM}3 donkey anti-rabbit (The Jackson Laboratory) antibodies were used as secondary antibodies. Nuclear staining was performed with 1,5-bis{[2-(di-methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9,10-dione (DRAQ5) (Cell Signaling). Images were captured using a Zeiss LSM 5 confocal microscope and analyzed with Zen software (Carl Zeiss) and ImageJ software.

Analysis of Gene Expression by Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated from 3 x 10^6 cells using TRIzol (Invitrogen), according to the manufacturer's instructions, and quantified by spectrophotometry. After oligo (dT)-primed reverse transcription of 1 μg of total RNA, the resulting cDNA was amplified using the primers listed below. Real-time quantitative PCR (qRT-PCR) analysis was performed with an automated rotor gene system RG-3000A (Corbett Research). The PCR reaction mix (20 μl) was composed of 10 μl QPCR sybr master mix (Finnzymes), 5 μl of diluted cDNA (each sample in triplicate) and a final concentration of 0.3 μM of each primer. Hypoxanthine guanine phosphoribosyl transferase (HPRT) primers were used as an internal standard. The following primers were utilized: human HPRT sense: 5′-GCTATAAATTCTTTGCTGACCTGCT-3′, antisense: 5′-ATTACTTTTATGTCCCCTGTTGACTG-3′; human heparanase sense: 5′- GTTCTAATGCTCAGTTGCTCCT−3′, antisense: 5′-ACTGCGACCCATTGATGAAA-3′; mouse HPRT sense: 5′-GTC GTG ATT AGC GAT GAA-3′, antisense: 5′-CTC CCA TCT CCT TCA TGA CAT C-3′; mouse heparanase sense: 5′-ACT TGA AGG TAC CGC CTC CG-3′, antisense: 5′-GAA GCT CTG GAA CTC GGC AA-3′; mouse COX-2 sense: 5′-GGG TGT CCC TTC ACT TCT TTC A-3′, antisense: 5′-TGG GAG

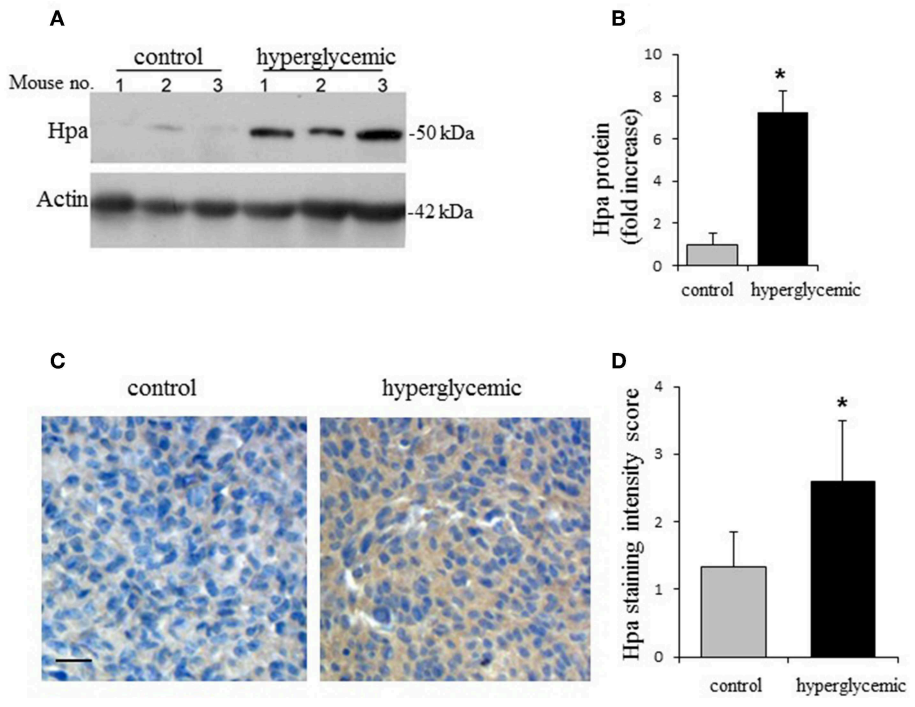

FIGURE 2 | Hyperglycemic conditions induce expression of heparanase in Panc02 pancreatic carcinoma *in vivo*. **(A,B)** Heparanase protein (Hpa) levels in the Panc02 tumors derived from control (normoglycemic) and hypeglycemic mice. **(A)** Lysates of tumor tissue were analyzed by immunoblotting. **(B)** The band intensity was quantified using ImageJ software; intensity ratio for Hpa/actin is shown, error bars represent ±SD. Two-sided Student's *t*-test $^*p < 0.02$; $n \geq 3$ mice per condition. **(C)** Immunostaining (brown) of Panc02 tumor tissue sections with the anti-heparanase antibody diluted (1:200) in CAS-Block was performed as described in section Materials and Methods. Scale bar: 50 μm. **(D)** Sections were scored in a blinded fashion according to the heparanase staining intensity (low/no staining = 1; medium staining = 2; high staining = 3). $n \geq 5$ mice per condition, at least 5 fields per tumor section were analyzed. The data shown are the mean ±SD of staining scores. Two-sided Student's *t*-test $^*p = 0.03$.

GCA CTT GCA TTG A-3′; mouse IL-6 sense: 5′- AGC CAG AGT CCT TCA GAG AGA TAC-3′, antisense: 5′- GCC ACT CCT TCT GTG ACT CC-3′, mouse TNF-α, sense: 5′-CAT CTT CTC AAA ATT CGA GTG ACA-3′, antisense: 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′.

Statistical Analysis

The results are presented as the mean ±SD or ±SE. $P \leq 0.05$ were considered statistically significant. Statistical analysis was performed using unpaired Student's *t*-test. All statistical tests were two-sided.

RESULTS

Dysregulation of Glucose Metabolism Accelerates PDAC Progression and Induces Heparanase Expression

Deterioration in glycemic control is characteristic of PDAC: hyperglycemia has been repeatedly observed in majority (according to some reports—up to 85%) of PDAC patients (2–4); and epidemiologic studies report increased incidence of pancreatic carcinoma in diabetic populations (6, 7). Thus, to investigate heparanase regulation in PDAC under dysregulated glucose metabolism, we utilized a murine experimental system, based on Panc02 mouse pancreatic carcinoma cells growing in C57BL/6J mice with the diet-induced metabolic syndrome, as described in Methods. Diet-induced metabolic syndrome in male C57BL/6J mice represents a reliable model, which closely parallels metabolic abnormalities in diabetic patients, such as increased circulating concentrations of glucose, hyperinsulinemia and impairment of glucose tolerance (47–49). Following 12 weeks of the diet intake, metabolic syndrome/impaired glucose metabolism was documented in experimental mice fed with high fat diet (but not in the control mice fed with the regular diet), as manifested by hyperglycemia, significantly increased glycated hemoglobin A1c (HbA1c) blood levels and glucose intolerance (**Figures 1A–C**), along with hyperinsulinemia (**Supplementary Figure 1**) and increased body weight. Then, both control (normoglycemic) and experimental (hyperglycemic) mice were injected subcutaneously with Panc02 cells, as described in Methods. In agreement with previous reports (51, 52), growth of Panc02 pancreatic carcinoma *in vivo* was markedly accelerated in hyperglycemic mice (**Figure 1D**). Additionally, tumors grown in hyperglycemic mice expressed markedly elevated levels of phospho-AKT [pAKT] (**Figure 1E**), one of the hallmarks of the PDAC tumorigenesis (53, 54).

We next compared heparanase expression in Panc02 tumors grown in experimental vs. control groups, applying immunoblotting. As shown in **Figures 2A,B**, markedly increased levels of heparanase protein were detected in Panc02 tumors growing in hyperglycemic, as compared to control (normoglycemic) mice. In agreement, quantitative RT-PCR analysis revealed ∼2-fold increase in heparanase mRNA levels in hyperglycemic vs. control mice. Additionally, immunostaining of the mouse tumor tissues with heparanase antibody revealed that Panc02 carcinoma cells, rather than host-derived stromal cells, represent the main source of the enzyme in tumors growing in hyperglycemic mice (**Figures 2C,D**).

AGE Induces Heparanase Expression in PDAC Cells *in vitro*

It was previously shown that various components of the diabetic milieu, including high glucose, free fatty acids, AGE, inflammatory cytokines (IL-6, TNF-α), upregulate heparanase in cells of non-pancreatic origin (29–31, 33–35, 50, 55). Importantly, increased levels of the aforementioned diabetic milieu constituents are present in the mouse model of metabolic syndrome/diabetes, used in our study (42, 47, 56–58). We therefore tested effects of various diabetic milieu components on heparanase expression in Panc02 cells *in*

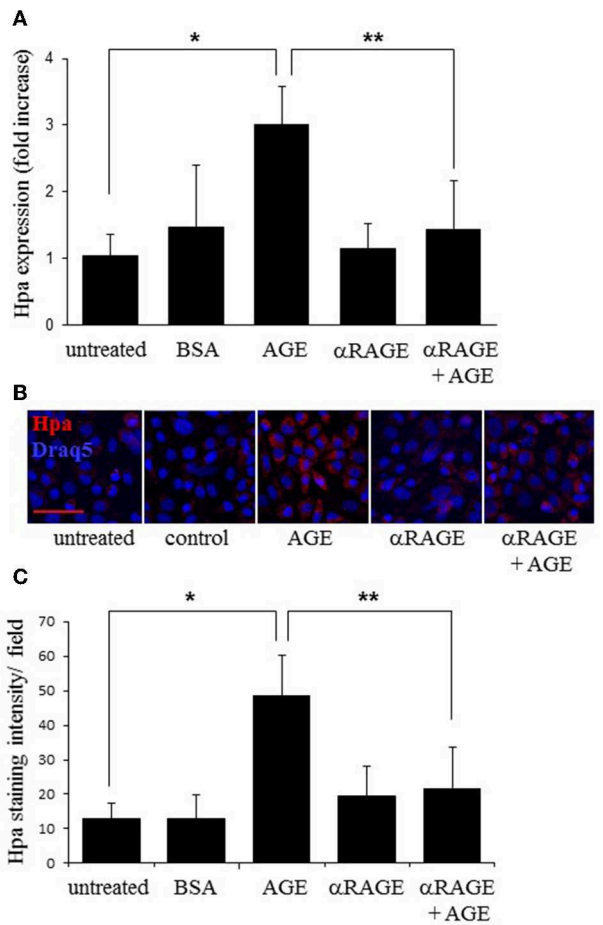

FIGURE 3 | Heparanase upregulation *in vitro* is mediated by AGE/RAGE signaling. **(A,B)** Panc02 cells were either untreated, or incubated (24 h, 37°C) with 200 μg/mL of AGE or BSA (control), with or without pretreatment with RAGE neutralizing antibody (αRAGE, 12 μg/mL) and analyzed for heparanase (Hpa) mRNA expression by quantitative real time PCR **(A)** and immunostaining **(B)**. Error bars represent ±SD. Two-sided Student's *t*-test $^{*}p < 0.008$, $^{**}p < 0.001$. **(B)** Cell nuclei were counterstained with DRAQ5 (blue). Scale bar: 50 μm. **(C)** Heparanase staining intensity was quantified using ImageJ Software per microscopic field, based on at least 4 fields per condition. Data shown are the mean staining intensity per 0.03 mm^2 microscopic field. Error bars represent ±SD. Two-sided Student's *t*-test $^{*}p < 0.002$, $^{**}p < 0.004$.

vitro. While treatment with either high glucose, fatty acids, insulin, or IL-6 failed to induce the enzyme expression (**Supplementary Figure 2**), treatment with AGE significantly increased expression of heparanase mRNA in Panc02 cells (**Figure 3A**). Immunofluorescent staining analysis also demonstrated increased heparanase protein levels in Panc02 cells following AGE treatment *in vitro* (**Figures 3B,C**), echoing *in vivo* observations (**Figures 2C,D**). Similar increase in heparanase expression in the presence of AGE was revealed in human PDAC cell lines MIA PaCa-2 and PANC-1 (**Supplementary Figure 3**).

AGE, whose formation is particularly augmented in diabetes due to combined effects of hyperglycemia and oxidative stress (39, 40), interact with the receptor for advanced glycation end products [RAGE], a multiligand receptor, expressed by numerous cell types, including PDAC cells (59, 60). Additionally, AGE are among the endogenous ligands known to activate toll-like receptor 4 (TLR4) (61–63), which is also expressed by PDAC cells (64, 65). As reported in Vaz and Andersson (64) and Kang et al. (65) and confirmed by qRT-PCR, Panc02 cells express both RAGE and TLR4. Responsiveness of Panc02 cells to AGE stimulation was further supported by upregulation of IL-6 and COX-2 following AGE treatment (**Supplementary Figures 4A,B**), as well as enhanced NFκB signaling, evidenced by increased levels/nuclear localization of phospho-p65 in AGE-treated Panc02 cells (**Supplementary Figure 4C**). Notably, it was previously shown that NFκB is involved in heparanase up-regulation in PDAC cells (66). Since NFκB activation is a known consequence of either TLR (67) or RAGE (68, 69) signaling, we next applied inhibitory approach to distinguish between these two pathways. While presence of TLR4-specific inhibitor TAK242 (70) did not affect heparanase induction by AGE in our system (**Supplementary Figure 5**), presence of RAGE-neutralizing antibody significantly decreased AGE-mediated heparanase induction, both at the mRNA and protein level (**Figures 3A–C**).

DISCUSSION

Among six cancer types attributable to diabetes (71), PDAC and diabetes display a unique reciprocal connection: PDAC is a presumed cause of derangement in glucose metabolism in a large number of cases, while diabetic state is known to promote pancreatic tumor progression (3, 4, 11, 71). Diabetes and PDAC are two heterogeneous diseases with a tremendous impact on health: PDAC has the lowest 5-year relative survival rate compared with all other solid tumor malignancies (1) and diabetes has become a pandemic (72). Thus, identification of pathways linking PDAC and impaired glucose metabolism is of high importance.

Here, applying mouse model of metabolic syndrome/diabetes and concurrent pancreatic carcinoma, we show that diabetic state leads to induction of heparanase expression in PDAC (**Figures 1**, **2**). This induction appears to be driven by AGE (**Figure 3**), a well-characterized member of the diabetic milieu. It should be noted that the limitation of the present study is that the single model was used for *in vivo* confirmation—due to enormous complexity of both diseases (PDAC and diabetes) it remains extremely challenging to establish additional mouse models faithfully reflecting concurrent pancreatic tumor progression and diabetes.

Given abundant evidence implicating heparanase in PDAC pathogenesis/aggressiveness/therapy resistance (13–17, 25), our finding may provide a partial explanation for the mechanism through which diabetic state contributes to pancreatic carcinoma progression. Indeed, elevated levels of the enzyme have been found in PDAC tissue samples (13) and in body fluids of patients with active pancreatic cancer disease as compared to healthy donors (16). Pancreatic cancer patients whose tumors exhibit high levels of heparanase mRNA had a significantly shorter postoperative survival time than patients whose tumors contained relatively low levels (13, 15, 73). Heparanase is a highly significant independent variable for lymph node metastasis in pancreatic cancer patients, further supporting crucial involvement of the enzyme in PDAC progression (14). Importantly, the aforementioned epidemiological observations are backed by the experimental data demonstrating accelerated tumor growth/increased invasiveness in PDAC cells engineered to over-express heparanase (13, 15, 26), as well as a reduction of primary tumor progression/metastasis in murine models of PDAC following administration of heparanase-inhibiting compounds (25, 74). Although several mechanisms controlling expression of the enzyme in various tissues have been described (30, 31, 55, 75), regulation of heparanase induction in PDAC remained under investigated.

To promote PDAC development heparanase acts through augmented release of HS-bound growth factors, removal of extracellular barriers for invasion (13–16, 25) and creation of tumor-stimulating "aseptic" inflammatory conditions, i.e., increased production of IL-6 (a key cytokine driving pancreatic tumorigenesis) by heparanase-stimulated tumor associated macrophages (TAM) (26). In agreement with this mode of action, we found significantly increased levels of IL-6 (and in accordance—increased TAM infiltration) in heparanase-overexpressing Panc02 tumors derived from hyperglycemic mice (**Supplementary Figure 6**). Additionally, ability of the enzyme to augment insulin/insulin-like growth factor 1 receptor signaling (76, 77), along with the well-documented hyperinsulinemia in PDAC patients [either in the setting of new inset or long-standing diabetes (3, 8, 9)], suggests that in heparanase-rich PDAC microenvironment insulin is expected to induce stronger pro-tumorigenic response. Thus, heparanase induction appears to be a part of the mechanism(s) through which diabetic state promotes PDAC and renders it highly aggressive, therapy-resistant and associated with particularly poor prognosis (3, 4, 10–12).

On the other hand, emerging involvement of heparanase in diabetes, including its role in the islet/beta cell damage (32, 78–80), taken together with augmented production of the enzyme by pancreatic carcinoma cells under hyperglycemic conditions (this study), implies that the enzyme may exacerbate PDAC-associated diabetes. Indeed, pioneering studies by C R. Parish and his group identified multiple

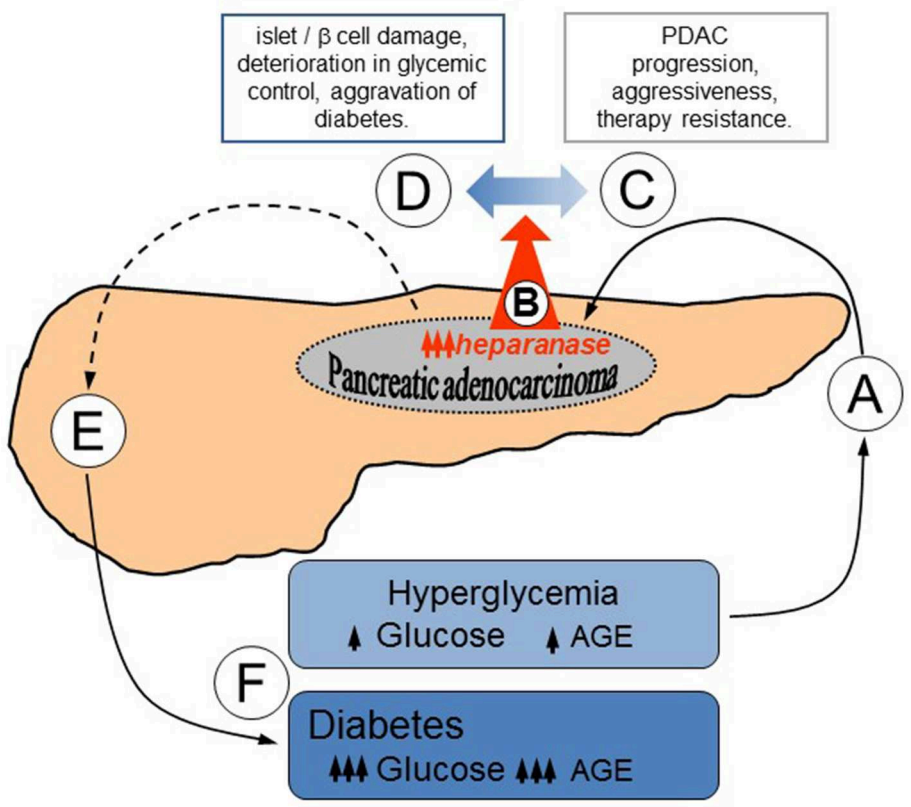

FIGURE 4 | Proposed mode of heparanase action in sustaining bidirectional relationship between PDAC and diabetes. **(A)** Hyperglycemic state, which occurs in the majority of PDAC patients (2–4), results in heparanase overexpression in carcinoma cells via AGE-dependent mechanism **(B)**. **(C)** Elevated levels of heparanase promote PDAC development through several well-defined mechanisms, including augmented release of HS-bound growth factors, removal of extracellular barriers for invasion, and creation of tumor-stimulating inflammatory conditions (13, 15, 25, 26, 74). **(D)** In parallel, contribution of heparanase to the islet damage [originally described in the setting of type 1 diabetes (32, 78–80), but highly relevant to the pathogenesis of PDAC-associated diabetes as well] may impair beta cell function **(E)**, exacerbating diabetic state. **(F)** Aggravation of diabetes further escalates AGE production, advancing PDAC heparanase expression and its protumorigenic action.

roles for heparanase in islet damage (originally—in the setting of type 1 diabetes) (32, 78–80). The islet-damaging heparanase actions include promotion of the leukocyte migration from pancreatic blood vessels and their passage across the islet basement membrane, as well as depletion of heparan sulfate which is required for beta cell survival (32, 78–80).

Importantly, beta cell damage, islet inflammation and islet-infiltrating leukocytes (particularly, macrophages) appear to promote type 2 diabetes (T2D) as well (81). Along with insulin resistance, beta cell dysfunction is a major component of T2D pathology, and clinical onset of T2D does not occur until beta cells fail to secrete sufficient insulin to maintain normoglycemia in the face of insulin resistance (81–85). Macrophages infiltrate islets in clinical and experimental T2D (86, 87) and are causally involved in beta cell dysfunction (81, 85, 88). Of note, patients with PDAC-associated diabetes often have high insulin levels and marked peripheral insulin resistance, similar to T2D [reviewed in (3)]. PDAC-associated diabetes also shares with T2D temporal relationship between insulin resistance, beta-cell dysfunction and development of impaired glucose tolerance (89): at earlier stages beta cells compensate for insulin resistance by increased insulin secretion, but progressive damage to beta cells leads to their dysfunction, deterioration in glycemic control, and at the later stage eventually leading to diabetes.

Given the secreted nature of the enzyme and its involvement in beta cell injury [via depletion of heparan sulfate (32, 78–80) and through tissue-damaging effects of the adversely-activated islet-infiltrating macrophages, similarly to those demonstrated in other pathologies (17, 26, 28, 50, 90)], it is conceivable that in hyperglycemic patients elevated levels of heparanase, originating from the tumor of exocrine pancreas (i.e., PDAC), can exert pathogenic effects within the endocrine compartment (i.e., islets), further impairing glucose metabolism and leading to the onset/aggravation of diabetes.

Thus, our study not only reveals the mechanism of heparanase upregulation in PDAC, but also implies that the enzyme may contribute to a self-reinforcing sequence of events underlying bi-directional association between diabetes and PDAC (**Figure 4**): hyperglycemic state, that occurs in the majority of PDAC

patients (**Figure 4A**), leads to heparanase overexpression in carcinoma cells via AGE-dependent mechanism (**Figure 4B**); increased levels of heparanase, in turn, promote PDAC progression (**Figure 4C**) through several previously-described mechanisms (13, 15, 25, 26, 74). In parallel, heparanase is capable of facilitating islet damage (**Figure 4D**), thus leading to beta cell dysfunction (**Figure 4E**), aggravating diabetic state and escalating AGE production, which further enhances PDAC heparanase expression and its protumorigenic action (**Figure 4F**).

Reciprocal relationships between PDAC and diabetes are certainly multifactorial in origin, and an array of molecular/cellular events underlying these relationships is far from being fully elucidated. Yet, our findings help to recognize the multilevel control that heparanase provides to heterotypic interactions among exocrine, endocrine and immune compartments of the pancreas in PDAC-diabetes link, suggesting that disruption of reciprocal causality between diabetes and PDAC through heparanase-targeting approaches may be of clinical benefit.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Hebrew University Institutional Animal Care and Use Committee, Hebrew University of Jerusalem, Israel.

AUTHOR CONTRIBUTIONS

RG, EH, AR, AA, and DN conducted the experiments. AM and AG acquired the data. RG, AM, EH, and ME analyzed the data. TP and ME designed the studies. AM, AG, and TP reviewed the manuscript. ME was responsible for conceptualization, research design, supervised the study, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01405/full#supplementary-material

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REVIEW
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Cancer Metastasis: The Role of the Extracellular Matrix and the Heparan Sulfate Proteoglycan Perlecan

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Cancer metastasis is the dissemination of tumor cells to new sites, resulting in the formation of secondary tumors. This process is complex and is spatially and temporally regulated by intrinsic and extrinsic factors. One important extrinsic factor is the extracellular matrix, the non-cellular component of tissues. Heparan sulfate proteoglycans (HSPGs) are constituents of the extracellular matrix, and through their heparan sulfate chains and protein core, modulate multiple events that occur during the metastatic cascade. This review will provide an overview of the role of the extracellular matrix in the events that occur during cancer metastasis, primarily focusing on perlecan. Perlecan, a basement membrane HSPG is a key component of the vascular extracellular matrix and is commonly associated with events that occur during the metastatic cascade. Its contradictory role in these events will be discussed and we will highlight the recent advances in cancer therapies that target HSPGs and their modifying enzymes.

Keywords: cancer metastasis, heparan sulfate proteoglycan, perlecan, heparanase, therapeutic

CANCER METASTASIS

Metastasis of a tumor is the systemic dissemination and colonization of tumor cells from the primary tumor to a secondary site and is a major cause of cancer-related deaths (1). Cancer is a global epidemic with an estimated 18.1 million new cases and 9.6 million deaths occurring in 2018 (2). Metastasis is an inherently inefficient process, that involves spatial and temporal regulation by both intrinsic and extrinsic factors. It is generally assumed that a cancer cell's genetic mutational burden compounds with advancing malignancy, resulting in the acquisition of proliferative and invasive traits, and finally the capacity to metastasize and colonize, distant organs. However, mutational burden alone does not fully explain the capacity of cells to invade, disseminate, and metastasize to secondary sites (3–6). The role of the microenvironment is now becoming appreciated as a key element in cancer progression, which is driven by interactions between tumor cells and their microenvironment (7–9).

The extracellular matrix (ECM) is a non-cellular meshwork of crosslinked macromolecules including collagens, proteoglycans, and glycoproteins, that form a dynamic, supramolecular, scaffold. It provides cues, both physical and chemical, which influence cancer progression

and metastasis. Biochemical and biomechanical cues present in the ECM, such as sequestered growth factors, ECM biomechanics and ultrastructural organization, are sensed by cells and converted into downstream cellular responses. These downstream cellular responses act in concert to alter malignant progression. Modulation of ECM components, by way of disrupted turnover, and aberrant or absence of post-translational modification (10), are some of the changes common to many diseases, including cancer (11, 12). Moreover, the ECM is a highly ordered structure, and its functional properties are contingent upon the precise assembly of ECM components (13). Subtle changes in the stoichiometry of these components may have downstream biological ramifications which affect tissue function. Cancer associated fibroblasts (CAFs) are important stromal cells within the tumor microenvironment that can be educated and/or recruited by tumor secreted factors. The capacity of CAFs to synthesize and remodel ECM components critically effects tumor progression (14). Understanding the nature of the heterotypic interactions between tumor cells, the ECM, and CAFs within the tumor microenvironment will offer insights into the mechanisms underpinning tumor progression and metastasis.

The process of metastasis is typically represented as a series of interconnected, and overlapping events, whereby certain conditions must be met before tumor cells transition to the next stage (**Figure 1**). These events include invasion into adjacent tissue, intravasation into the bloodstream and lymphatics, cancer cell survival during transit and extravasation out of vessels, and finally secondary organ colonization. The ECM is a key component throughout this cascade of events, with its involvement in modulating the behavior of both tumor and non-malignant stromal cells at all steps along the metastatic cascade.

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is one of the key programs in cancer that is thought to facilitate the shift in tumor cell behavior from a static epithelial phenotype to a more migratory, invasive, and mesenchymal one (**Figure 1**, Box 1). EMT and its regulatory signaling pathways are influenced by biochemical cues within the ECM. For instance, ECM environments rich in the glycosaminoglycan (GAG) hyaluronan (HA), transduce signals through the membrane receptor CD44, triggering EMT (15–18). The glycoprotein tenascin C has also been shown to be elevated in late stage mammary invasive ductal carcinomas at the tumor-stromal border. Here, it induces EMT through the proto-oncogene tyrosine-protein kinase Src and focal adhesion kinase (FAK) axis (19, 20). Furthermore, the shift in expression of the heparan sulfate proteoglycan (HSPG) syndecan-1 from tumor cell expression to stromal cell expression (*viz*. vimentin positive CAFs) has been shown to feed back onto cancer cells and drive EMT in many solid tumors (21). However, in contrast, Shen et al. (22) demonstrated that tubulointerstitial nephritis antigen-like 1 (TINAGL1), an ECM protein which competitively binds to integrins α5β1, αvβ1, and epidermal growth factor receptor (EGFR), can inhibit fibronectin-mediated FAK/EGFR signaling. This highlights how the balance between multiple ECM molecules can regulate the same intracellular signaling networks.

Invasion and Intravasation

Tumor cell invasion is initiated through the breakdown of the interactions (i.e., cell-cell and cell-ECM) at the primary tumor site, allowing cells to invade into the adjacent tissue (**Figure 1a**), in conjunction with local remodeling of the adjacent basement membrane (BM). As tumor cells pass through the local microenvironment of the primary site, they are exposed to a milieu of biomechanical cues within the ECM such as tissue stiffness, density and porosity (23–25), which regulate tumor cell fate. Seminal work demonstrated the ECM's importance at initial stages of metastasis, where interactions between tumor cells and a fibrotic and stiff extracellular matrix induced a malignant and invasive phenotype, which could be blocked to re-establish tissue order (26). At the tissue organizational level, the alignment of collagen fibers has been shown to have prognostic value in breast cancer whereby collagen fibers aligned perpendicular to the tumor periphery, known as tumor-associated collagen signature-3 (TACS-3), are prognostic of patient survival (27, 28).

Hydration of tumor tissue is strongly influenced by the presence of specific glycosaminoglycans (GAGs) within the tissue, due to their anionic structure and their ability to attract water. As hydration increases, increased intra-tumoral hydrostatic pressure rises and alters the biomechanical properties of the tissue which is known to be crucial to invasiveness (29, 30). Perfusion of nutrients, growth and chemotactic factors are also affected leading to changes in cancer cell invasion (31). Finally, matrix metalloproteinases (MMPs) released from both tumor and stromal cells degrade the ECM and facilitate local invasion (32, 33). The release and activation of ECM-sequestered growth factors [e.g., transforming growth factor (TGF)-β, fibroblast growth factors (FGFs)] may also play a part in this malignant process (34).

Following local invasion at the primary site, tumor cells typically spread around the body via the hematogenous or lymphatic networks which requires traversing the vascular and/or lymphatic BMs (**Figure 1b**, Box 2). However, tumors need not be clinically advanced for this to occur, as dissemination has been observed very early in tumor formation, even before clinical symptoms of disease are evident (35, 36). BMs are specialized tissues underlying epithelial and endothelial structures. BMs are membrane like structures with low porosity and their constituents are densely arranged together. Thus, for cells to traverse BMs, known as intravasation, they require the activation of specific protease-dependent and -independent programs (37–39). BMs impart polarity and survival signals to cells in contact with them, in addition to acting like a molecular sieve for the perfusion of nutrients and molecules from the blood through to the interstices. As such, the structural integrity of vessels and their BMs presents a major obstacle to invading tumor cells. However, in cancer, disruption of BMs is commonly observed. A series of recent studies (40, 41) demonstrated that the ECM molecule hyaluronan and proteoglycan link protein-1 (HAPLN1) decreased with aging of the ECM. This resulted in disruption of the vascular BM and increased vessel permeability, leading to subsequently enhanced

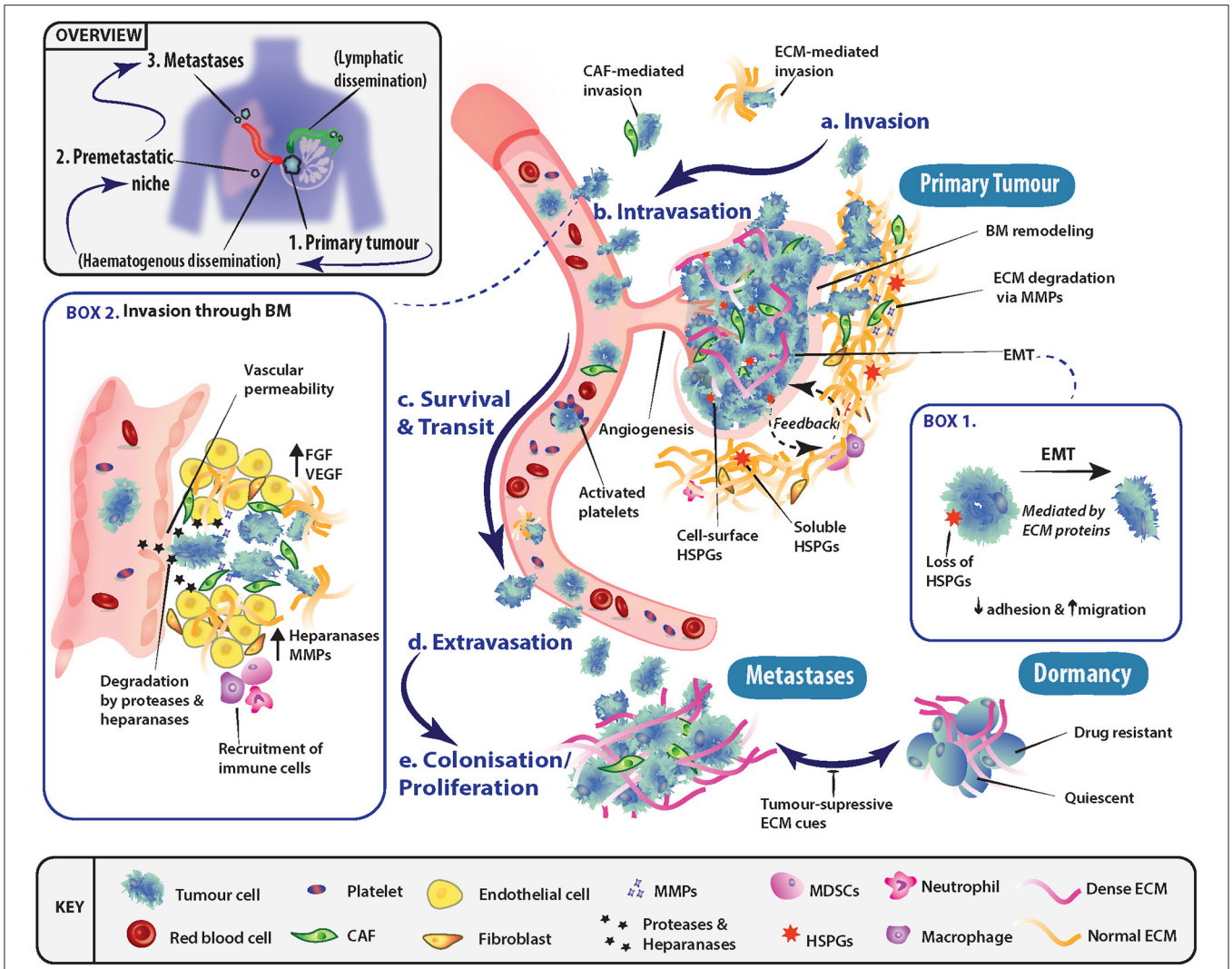

FIGURE 1 | Role of the extracellular matrix in driving progression through stages of the metastatic cascade. **(a)** Primary tumor cells may undergo epithelial-mesenchymal transition (EMT) (Box 1) and invade through basement membranes (BM) into the surrounding stroma. Tumor cell local invasion and metastatic dissemination is often facilitated by cancer-associated fibroblasts (CAFs) or specific ECM components, which may enhance invasion or modulate the immune system. **(b)** To disseminate to a secondary site, tumor cells must access the vascular system and intravasate through the endothelial BM. This occurs in part through the release of proteases and heparanase, which disrupt BM integrity (Box 2). **(c)** The circulating tumor cells (CTCs) must then survive transit to secondary sites of metastasis and can be assisted by platelet activation as well as accompanying CAFs. **(d)** To exit the vessel, cells extravasate into the surrounding tissue and seed at distinctly different tissues from the primary tumor. Overt colonization of secondary sites by disseminating tumor cells (DTCs) is greatly enhanced through extravasation at premetastatic niches. **(e)** Extravasated cancer cells typically have three fates, either colonize and proliferate to form overt metastases, enter a reversible state of dormancy or, in most cases, die.

melanoma metastasis in mice. In addition, HA has been shown to be important in the regulation of vascular endothelial barrier permeability, through stabilization of cell-cell junctions (42, 43). Furthermore, high molecular weight HA secreted by tumors has been shown to negatively regulate hyaluronan binding protein 2 (HABP2), a serine protease, which is known to compromise vessel integrity (44). Along with the release of proteases by tumor cells, invasion through BMs can be affected by the release of heparanase (45, 46), which degrades the HS chains of HSPGs located in the BM and ECM, as reviewed by (47) (**Figure 1**, Box 2).

Survival and Transit Through the Circulatory System

Once tumor cells enter the circulation, their survival in the absence of cell-cell and cell-ECM cues is a crucial factor determining metastatic outcome (**Figure 1c**). Various mechanisms have been uncovered which facilitate cancer cell survival in the circulation. For example, circulating tumor cell (CTC) clusters exploit mechanisms such as tropomyosin receptor kinase B (TrkB) signaling to combat apoptosis induced by the lack of cell-ECM interactions, termed "anoikis" (48, 49). In addition, the close association of stromal elements with tumor

cells in circulation, namely CAFs and their secreted factors (e.g., FGFs) enhance survival and facilitate metastasis (50). Platelet derived TGF-β signaling also protects against the lack of cell-ECM interactions present in circulation, through inducing a mesenchymal-like phenotype (51). The activation of platelets provides CTCs with fibrinogen (52) and tissue factor (53), which protects against immune clearance within the circulation and at secondary sites. The cues provided may temporarily be substituting for the absence of correct tissue and ECM contacts, and therefore likely provide survival signals that protect cancer cells (7).

Extravasation

Tumor cells that survive within the circulation and lodge in the vasculature of secondary organs, must extravasate into the parenchyma in order to begin the colonization process (**Figure 1d**). The site of extravasation may be determined to some extent by the formation of "pre-metastatic niches" (54), which can in part explain metastatic organotropism (55). Of note, secreted factors from the primary tumor, such as MMP-3, -9 and -10 (56, 57), can induce the production of vessel destabilizing factors at secondary sites of future metastasis, which act to enhance extravasation. Once extravasated into secondary organs, tumor cells must adapt to the new local cues (i.e., ECM molecules as well as locally secreted growth factors) in order to persist and go on to form overt metastases (**Figure 1e**). At this stage, the alternatives are entry into a dormant state, or ultimately death. Therefore, this phase in the cascade relies on the interaction between the extravasated tumor cells and the characteristics of the host tissue microenvironment for the successful establishment and outgrowth of overt metastases.

Secondary Organ Colonization

More recently, it has become increasingly apparent that secondary sites may not simply be naïve recipients of disseminated cells, and instead, the ECM and local microenvironment may be remodeled prior to the arrival of tumor cells. This concept has been termed the pre-metastatic niche (54, 58–60), and encompasses the idea that primary tumors were capable of remodeling the tissue microenvironment of secondary organs prior to their arrival in order to facilitate metastatic colonization (**Figure 1**). This was first demonstrated by Kaplan et al. (60) who showed that bone marrow derived hematopoietic progenitor cells, activated by secreted factors from the primary tumor, are capable of remodeling secondary lung tissue to produce a fibronectin-rich environment prior to tumor cell arrival. This environment then acts to support overt colonization by the seeding tumor cells. Cell-ECM interactions not only supply an anchorage point for seeding, but also activate survival and proliferative signaling programs transduced through integrin complexes and their associated downstream signaling (61–63). These cell-ECM interactions, and signaling networks are potential targets for therapeutic intervention, such as has recently been shown for ROCK inhibition (64, 65). CTCs arriving in secondary organs typically initiate and drive ECM remodeling at these sites. For example, breast cancer cells metastasizing to the lung produce their own tenascin C that promotes survival and macrometastatic outgrowth via NOTCH and WNT stem cell pathways (66). This is further perpetuated by secretion of TGF-β by cancer cells, which stimulates fibroblasts to secrete periostin (POSTN), further activating WNT signaling (67). Additionally, when secreted at elevated levels, bone morphogenic protein (BMP)-4 and -7 have been demonstrated to cause cancer cell dormancy in both lung (68, 69) and bone (70), which is driven by secreted protein acidic and rich in cysteine (SPARC) in the prostate cancer setting (71).

Another example of ECM induced dormancy has been observed within the "perivascular niche," which, in some tissues, such as bone and lung, produce a source of quiescing thrombospondin 1 (TSP1) (72). Upon vascular disruption, in situations such as inflammation or wounding, TSP1 secretion is disrupted and the generation of a tumor-promoting microenvironment ensues and facilitates metastatic outgrowth (72–74). Additionally, vascular endothelial cell secretion of perlecan has also been shown to influence lung cancer cell dormancy in the perivascular niche (75). Perlecan has also recently been shown to be upregulated in CAFs in pancreatic cancer through secretion of TNFα from p53 gain-of-function (but not p53 loss-of-function) cancer cells. Cancer cell education of CAFs and the elevated secretion of perlecan was responsible for the generation of a prometastatic microenvironment (76).

It is clear that the ECM is a key regulatory determinant of tumor cell phenotype and behavior, which is dynamically modified throughout the different stages of metastatic progression. The inherent nature of a patient's ECM and the particular modifications accrued by the ECM throughout tumorigenesis may be viewed as either necessary and/or sufficient to enable malignant progression. Thus, the tumor ECM represents a vast territory of underexploited therapeutic targets in treating cancer and cancer metastasis.

PROTEOGLYCANS AND THEIR GLYCOSAMINOGLYCAN CHAINS

Glycosaminoglycans (GAGs) are well-established regulators in the metastatic spread of cancer (77–82). GAGs are negatively charged glycan structures comprised of repeat disaccharide units and belong to one of four subgroups: (1) heparin/HS, (2) chondroitin/dermatan sulfate (CS/DS), (3) keratan sulfate, and (4) hyaluronic acid or HA. All GAGs, other than HA, are covalently attached to the core protein of proteoglycans (PGs). HSPGs are ubiquitously expressed and consist of a protein core to which HS chains are covalently linked. Biological activities associated to HSPGs are mediated through interactions with various ligands, via the protein core or the HS side chains, where the specificity and affinity of these interactions is related to the HS chain structure and position of sulfate groups (83, 84). HSPGs are involved in multiple roles ranging from structural development and maintenance, to organization of the ECM and BM via binding with matrix molecules including collagen IV, fibronectin, and laminin (85, 86). In particular, HS modulates cell-cell interactions by acting as a co-receptor for different cell surface receptors as well as influencing cell-ECM interactions.

HS also mediates the sequestering of various growth factors, chemokines, cytokines, morphogens, and enzymes by forming protected "reservoirs" that upon release can promote receptor-ligand signaling complexes to mediate crucial regulatory roles in cellular processes to maintain tissue homeostasis (87). Structural modification of HS can occur post-translationally by the actions of sulfotransferases, sulfatases (Sulfs), heparanase. MMPs and other proteolytic enzymes (e.g., plasminogen) can modify the protein core of HSPGs and can therefore regulate HSPG-dependent signaling pathways (88, 89). Heparanase is the only mammalian derived enzyme that is capable of degrading HS (90) as well as heparin (91). HSPGs regulate a myriad of activities including; cell adhesion and migration, proliferation, differentiation and morphogenesis, vascularization, cytoskeletal organization, and tissue repair (92). These phenomena are essential for metastasis onset and success.

Heparan Sulfate Proteoglycans

HSPGs have intracellular, cell surface, and ECM localizations, including the BM (93). The BM PGs, perlecan, agrin, and collagen XVIII are primarily substituted with HS GAGs. Endothelial, epithelial, immune cells, and fibroblasts all synthesize these HSPGs, though HSPGs produced by different cell types will be decorated with HS chains that differ in structure, and thus their biological interactions will also differ (94). Hence, HSPGs have been reported to have both pro-angiogenic and anti-angiogenic properties due to heterogeneous HS structures and thus, their interactions with numerous growth factors differ (95). Cell surface HSPGs belong to members of the transmembrane syndecan (SDC) and the glycosylphosphatidyl-inositol (GPI)-anchored glypican (GPC) families. There are four mammalian SDCs (SDC 1-4) and six GPCs (GPC 1-6). The location of HS chains on the PG protein core with respect to the cell surface differs between SDCs and GPCs. The HS chains that decorate GPCs are located close to the plasma membrane. In the SDCs, the HS chains are located at sites further away from the cell surface. The SDC family members are differentially expressed on different cell types, SDC-1 is found on epithelial cells, SDC-2 on fibroblasts and endothelial cells, SDC-3 is on neural cells, and SDC-4 is ubiquitously produced by most cell types but in relatively low abundance (96). Shedding of cell surface HSPGs provides another mechanism to control HSPG distribution, as SDCs can be enzymatically released by MMPs, where GPCs are shed by GPI-specific lipases (97, 98). While, HSPG shedding downregulates their functions at the cell surface, the shed, and now soluble, HSPGs may facilitate the transfer of bound ligands to signaling receptors on neighboring cells conveying positive or negative effects in cancer progression (99). Opposing roles for anchored vs. shed GPCs have been demonstrated. Overexpression of GPC-3 in hepatocellular carcinoma (HCC) promotes tumor growth via WNT (100) and insulin-like growth factor (IGF) signaling (101). However, soluble GPC-3 blocks WNT signaling and inhibits HCC growth (102). Similarly, transmembrane GPC-1 promotes proliferation and metastatic growth of pancreatic cancer cells (103, 104), whereas, soluble GPC-1, inhibits the mitogenic response to FGF-2 and heparin-binding EGF-like growth factor (HBEGF) (104). Additionally, glycoproteins such as betaglycan and CD44v3 are part-time HSPGs, and may have potential roles in cancer (105, 106).

The strategic location of HSPGs in tissues are critical to their functional roles. Localization of SDCs and GPCs in the plasma membrane regulates intracellular and cell-ECM signaling. Localization of HSPGs in the BM regulates their barrier functions and co-ordinates cell-cell/ECM-cell interactions. Localization of perlecan at the interface of tissues and tissue layers, coupled with their sequestered growth factors, has been hypothesized as on-site "depots" that assist with the restoration of those borders when compromised (107). Cell surface HSPGs can also act as docking modules for MMPs (108, 109), which promote invadopodia and enable cells to move in specific directions through the ECM (110). MMPs secreted by invadopodia promote the invasion of breast carcinoma cells into the ECM (111). Endothelial cells also release granules containing MMP-2 and MMP-9 at focal sites, and their focal MMP activation can contribute to directed angiogenic events (112). It has been proposed that cell surface HSPGs generate a tract in the ECM for the migration of cells. Weak interactive properties between cells and HS allow the cell to "walk" along the cell surface or ECM HS chains facilitating cellular migration (108). Shed fragments of cell surface HSPGs can also influence cell proliferation by amassing in intracellular spaces and sequestering growth factors (86). Degradation of HS, by heparanase, on SDC-1 produces heparin-like fragments that activate FGF-2 mitogenicity (113). The biological role of a HSPG therefore depends on the properties of its protein core, the number of GAG chains attached, its localization in cells and tissues, as well as the biosynthetic modifications its GAG chains receive *in situ*.

The vast range of biological functions attributed to GAGs in cancer metastasis, and numerous other biological events, is due to their non-templated controlled, highly heterogeneous and complex structure, which enables the regulation of tissue-specific functions. Biosynthesis of GAGs is a sequential process that occurs in the endoplasmic reticulum and the Golgi apparatus (114). This process is governed by a large family of enzymes, and while the function of these enzymes is known, the process that controls specific GAG structure, as well as the degree and position of sulfate motifs is not. HS, the major GAG discussed herein, consists of a glucuronic acid-galactose-galactose-xylose-linker region (GlcA-Gal-Gal-Xyl) which is initiated by the enzymatic transfer of xylose to specific serine-glycine residues of core protein sequences (115). HS assembly occurs by sequential addition of N-acetyl glucosamine (GlcNAc) to the linkage tetrasaccharide acceptor, then GlcA to form GlcA-GlcNAc disaccharide repeats (**Figure 2**). As the chain polymerizes, HS is also enzymatically modified by sulfotransferases and an epimerase at various positions in a coordinated manner, with the product of one modification serving as substrate for the next step (116). The enzyme, N-deacetylase/N-sulfotransferase (NDST), substitutes the N-acetyl group with a sulfate group in between clusters of GlcNAc, leaving regions of the chain unmodified. Further modifications include; epimerization of GlcA to iduronic acid (IdoA) and 2-O-sulfation of IdoA, O-sulfation of GlcNS by sulfotransferases at C6 or less commonly, at C3. Thus, sulfation along HS chains is not uniform and contains highly sulfated

regions (NS domains) and largely unmodified regions (NA domains). Ligand binding to HS depends on the arrangements of NS and NA domains, and on the modified residues within the NS domains. The HS-FGF-2 interaction exemplifies a GAG-growth factor interaction and demonstrates how specific HS structures facilitate FGF-2/FGFR-mediated signaling. HSPGs play a vital role in the FGF-2/FGFR interactions by assembling FGF-2 near the receptor, which forms a ternary complex that stabilizes the ligand-receptor complex, thereby promoting signal transduction (117). HS chains require N-sulfated glucosamine and 2-O-sulfated IdoA units to bind to FGF-2 (118). At the same time, for HS chains to bind FGFR, they require 6-O-sulfated GlcN residues along with 2-O-sulfated IdoA with N-sulfated GlcN residues also reported to be involved in this interaction (119).

A number of studies have highlighted that HS dysregulation in cancer can occur when the expression and behavior of HS-synthesizing and HS-modifying enzymes are altered (120–124). For instance, Weyers et al. reported on the structural differences found in sulfation patterns between normal and breast cancer tissues in addition to differences in sulfation between patients with non-lethal and lethal cancer (121). Specifically, patients with lethal cancer presented with decreased levels of 6-O sulfation of HS, and increased levels of unsulfated disaccharides. Furthermore, observed increases in HS chain length suggested that the breast tissue underwent changes in the HS polymerization pathway. A similar study assessing transcriptional patterns in panels of breast, prostate, colon cell lines, and isolated tumors confirmed that changes in HS biosynthetic enzyme levels occurred in a tissue-specific manner and particularly affected modification enzymes which undertake HS sulfation (120); supporting previous studies in animal models (125, 126). Interestingly, the authors also discovered that there was no difference in the biosynthetic enzymes between normal and metastatic cell lines and proposed that the cells maintain relatively normal PG expression pattern at the cell surface in order to avoid immune detection.

The two known human orthologs of sulfatases (HSulf-1 and HSulf-2) are released as soluble enzymes capable of cleaving the 6-O sulfate on glucosamine (127). Despite similarities in their structural organization and mechanistic action, these sulfatases have been shown to have opposite roles in cancer progression. HSulf-1 suppresses FGF-2-mediated tumor cell proliferation and invasion, HSulf-2 augments these activities to progress disease, as examined in HCC (128). HSulf-1 is downregulated in breast, pancreatic, ovarian, head and neck cancers according to a tumor suppressor effect (129). HSulf-2 has additional roles in the pathogenesis of non-small-cell lung carcinoma (NSCLC), pancreatic cancer and glioblastoma despite unaltered expression levels (130, 131). In contrast, prostate cancer cells overexpressing HSulf-2 present with reduced levels of the trisulfated disaccharide UA(2S)-GlcNS(6S) in conjunction with an increase in EMT markers and WNT signaling (132). In this regard, the role of HS-modifying enzymes in regulating EMT is noteworthy, given its important role in metastatic progression (133, 134). For instance, Maupin et al. consistently found upregulation of the HSulf-2 enzyme in various *in vitro* models mimicking aspects of pancreatic cancer EMT (135). Furthermore, increased methylation of the HSulf-1 promotor was found to be present in samples from gastric cancer patients (55%) as compared to healthy patients (19%) (136). This was measured using cell-free serum samples taken from patients and the authors advised that methylation-induced silencing of HSulf-1 showed potential as an early diagnostic tool for cancer. Likewise, other studies have proposed that specific biosynthetic trends for each tumor type (121) or proteoglycan staining patterns based on associated GAGs could serve as potential prognostic biomarkers in various histological types (123). Certainly, this area of research will continue to evolve as new analysis tools become available to study GAG structure and identify key structure-function relationships. Significantly, tumor cells have been reported to actively manipulate the binding capacity of their HSPGs for FGF-2 and other growth factors, by modifying the overall density and sulfation pattern of their HSPGs (81). Since natural killer (NK) cells recognize particular HS fine structural patterns, explicitly 6-O-sulfonation and N-acetylation patterns, cancer cells can change their HS patterns to evade NK cells and immune surveillance (137, 138). Studies of breast and pancreatic cancer cells that express increased extracellular heparanase and aberrant HSulf activity have also been shown to affect recognition by NK cells (139).

The Role of Perlecan in Cancer Metastasis

Among the various contributory factors so far identified to be involved in the various stages of cancer progression, perlecan, a modular HSPG stands out as an important player. Perlecan contains multiple domains (**Figure 2**) which allows participation in a variety of roles, as well as being a major structural constituent of BMs (85, 107, 140–143). Perlecan is encoded by the HGPS2 gene, and is predominately substituted with HS chains, though depending on the cell type it originates from, it may be substituted with CS, DS, a combination of HS, CS, and/or DS, or as a GAG-free glycoprotein (144, 145). The N-terminal Domain I is most commonly decorated with three HS chains, whereas at the C-terminal, Domain V can also be substituted with HS and/or CS chains (146). The protein core is divided into five domains, with each domain involved in binding to various partners, from classical ECM components such as collagen IV, nidogen-1, and fibronectin, to growth factors, including FGF-2, -7, vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (85, 147, 148). While it is present in the BM of most endothelial and epithelial cells, perlecan also associates with the cell surface via interaction with α2β1 integrin (149). The c-terminal fragment of perlecan can exist as a separate fragment to the perlecan protein core, known as endorepellin, though it is not separately synthesized but rather is a result of proteolytic cleavage of secreted perlecan by proteases (150).

Interestingly, the two other HSPGs of BMs, agrin, and collagen XVIII, do not share much structural homology with perlecan, with the exception of Domain V of agrin (142). Although Domain I is unique to perlecan (151), it does contain the SEA (Sperm protein, Enterokinase, Agrin) module, which is present within other ECM proteins. GAG decoration on perlecan has been shown to be modulated by the presence of the SEA module since its deletion results in a recombinant protein

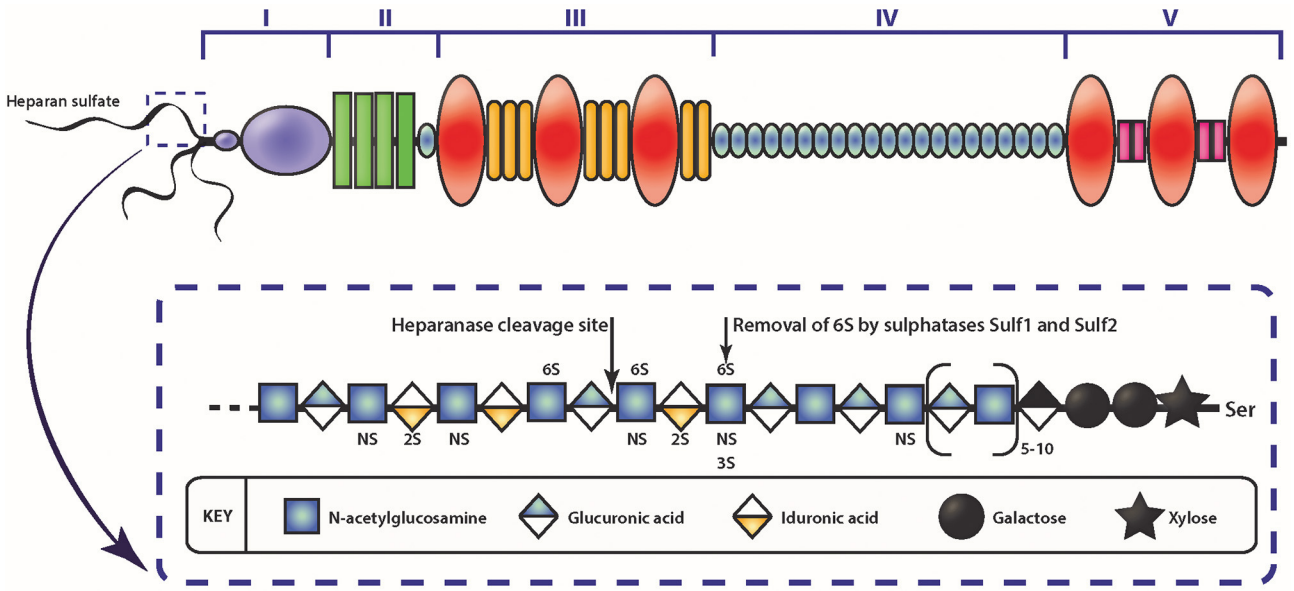

FIGURE 2 | Schematic diagram of the HSPG perlecan and HS. The different domains of perlecan are depicted by roman numerals. The insert depicts a schematic of HS represented by the repeating disaccharide of N-acetyl glucosamine and glucuronic acid (or iduronic acid) and sulfate moieties that can occur. Enzymatic modification of HS can occur via heparanase cleavage, resulting in smaller molecular weight fragments, or cleaving the 6-O sulfate on glucosamine via sulfatase.

with decreased HS content and an increase in CS (152). The importance of GAG decoration on perlecan has been further demonstrated in Hspg2$^{\Delta 3/\Delta 3}$ mice, whereby deletion of exon 3 of the Hspg2 gene removes the GAG attachment sites in Domain I and the mice presented with impaired angiogenesis, delayed wound healing, and retarded tumor growth (153). The functions that perlecan Domain I plays in various cellular functions cannot be overstated, most notably in angiogenesis (141–143, 154) and is predominantly due to the GAG chains that decorate this domain. The HS moieties of perlecan can bind a variety of pro-angiogenic factors including FGF-1, -2, -4, -7, -10, hepatocyte growth factor and TGF-β (85, 142, 154, 155). The pro-angiogenic activity of perlecan is achieved primarily through the interaction between HS, that decorate the protein core, FGF, and its corresponding receptors. These interactions actively coordinate cell proliferation, motility and adhesion (94, 156, 157). Conversely, and despite being a key region within a pro-angiogenic parent molecule, endorepellin is a potent inhibitor of angiogenesis (158, 159). Endorepellin, via the protein core, binds to both VEGFR-2 and α2β1 on endothelial cells triggering a signaling cascade that disrupts cell actin cytoskeleton and inhibits cell motility (149, 158, 160). Endorepellin is also reported to have transcriptional control by suppressing HIF-1α, a key transcription factor involved in promoting angiogenesis (159). Endorepellin is comprised of three laminin-like globular domains (LG1-LG3) with most of the biological activity attributed to LG3, cleaved from the parent molecule by protease digestion (161, 162). Circulating LG3 levels have been shown to be reduced in breast cancer patients and are being explored as a biomarker for cancer progression and invasion (163). The expression of perlecan has been investigated in various cancer types both *in vitro* and *in vivo* (**Table 1**). Although the findings are inconsistent, it is apparent that perlecan controls cancer progression by regulating interactions between cells and signaling molecules during the various stages, including ECM dysregulation, angiogenesis and invasion, which will be discussed in the following sections.

Extracellular Matrix Dysregulation

Cells interact with the ECM to regulate their activities and behavior. This interaction can occur directly through cell surface receptors, including integrins and discoidin domain receptors, and indirectly, via the release of growth factors and cytokines sequestered in the GAG chains (88, 178). ECM remodeling is instrumental to these essential functions including a fundamental role in angiogenesis (179). ECM remodeling removes the restrictive physical barrier, liberating endothelial cells to proliferate and migrate, which is coupled with the release of sequestered pro-angiogenic growth factors from HS chains of perlecan. The ECM is constantly deposited, remodeled, and degraded during development through to maturity to maintain tissue homeostasis (180, 181). Tissue inhibitor of metalloproteinase 3 (TIMP-3) inhibits ECM turnover and has been associated with cancer (182). This enzyme binds to sulphated GAGs on perlecan; further highlighting the significance of sulfation patterns in modulating protein activity (183). The highly dynamic nature of the ECM plays a crucial role in cancer progression and is the first barrier to developing metastasis. ECM remodeling is hijacked by tumor cells and invading stromal cells, resulting in dysregulated remodeling and dynamics (184, 185). This alters the composition and organization of the ECM and eventually leads to changes in its

TABLE 1 | Summary of *in vivo* observations for perlecan expression in various cancer types.

Cancer type	Assessment technique	Observations	References
Melanoma	Immunohistochemistry	Increased in BM at tumor-stroma interface and surrounding blood vessels Increased levels in tissue	(164)
	mRNA expression	Increased levels in tissue	(165)
Colon	Immunohistochemistry	Increased in stroma	(166)
Lung	Immunohistochemistry	Decreased to undetected in BM at tumor-stroma interface	(167)
	mRNA expression	Increased levels in tissue	(165)
Breast	Immunohistochemistry	Decreased to undetected in BM at tumor-stroma interface	(168, 169)
	mRNA expression (*in situ*)	Increased levels in tumor and stromal cells	
	Immunohistochemistry	Increased in stroma	(166)
Heptocellular carcinoma (HCC)	Immunohistochemistry	Increased in BM at tumor-stroma interface and blood vessels in stroma	(170)
	Immunoelectron microscopy	Increased at BM at tumor-stroma interface	
Intraheptatic cholangiocarcinoma (ICC)	Immunohistochemistry	Decreased to undetected in stroma	(171)
	mRNA expression (*in situ*)	Increased levels in tumor cells and stromal fibroblasts	
Ameloblastoma	mRNA expression (*in situ*)	Increased levels in stromal cells	(172)
Prostate	Immunohistochemistry	Increased in stromal cells	(173)
Ovarian	Immunohistochemistry	Decreased to undetected in BM at tumor-stroma interface Unaltered in BM of surrounding blood vessels or stroma	(174)
Pancreatic	Immunohistochemistry	Increased in BM and stroma	(175)
Oral squamous cell carcinoma (SCC)	Immunohistochemistry	Decreased to undetected in BM at tumor-stroma interface Increased in stroma	(176)
Glioblastoma	mRNA expression	Increased levels in tissue	(177)

essential properties (23, 25). However, the exact interactions and the role of BM components such as perlecan in mediating the abnormalities remain unstudied.

The breaching mechanism by which tumor cells invade the BM has not been clearly determined but has been proposed to involve a number of ECM-distinct and most likely complementary mechanisms: proteolytic degradation of the ECM in parallel with abnormal ECM synthesis (186). Degradation of ECM is mediated by multiple proteases including MMPs, ADAMs, and ADAM-TS (short for a disintegrin and metalloproteinase, and a disintegrin and metalloproteinase with thrombospondin motifs), in addition to heparanase, liberating pro-angiogenic factors that in turn activate angiogenesis and promote the proliferation of tumor cells (185, 187). Stromal cells, including CAFs, along with infiltrating immune cells and tumor cells, results in a sustained presence of these proteinases. This situation overall leads to the progressive destruction of normal ECM and establishment of the cancer-associated ECM. Remarkably, it is the same set of proteins, in different structural configurations and likely altered interactions with each other and the surrounding environment, that results in the abnormal ECM. Certain regions within the ECM have been identified to be important for tumor cell proliferation and survival but can be partially hidden or "cryptic;" only becoming unmasked upon enzymatic digestion (142). At present, no cryptic epitopes have been identified for perlecan but undoubtedly the fine structural sequences of the HS chains may be accountable.

Angiogenesis

Angiogenesis is a key requirement for cancer growth and progression (188); this multi-step process is dependent on ECM remodeling and endothelial cell activation for the coordinated differentiation into functional vessels. HSPGs have long been acknowledged to control angiogenesis via the sequestering and release of growth factors which regulate endothelial cells, smooth muscle cells, and fibroblasts (189). The role of perlecan in pro-angiogenetic and anti-angiogenic functions place it center stage. Both tumor cells and host stromal cells synthesize perlecan; confirmed by a series of early xenograft immunostaining and transcriptional studies (166, 168, 190). The secretion of perlecan by tumor cells was proposed by the authors to facilitate formation of blood vessels during tumor expansion through the binding and interaction between perlecan and angiogenic growth factors. The incorporation of tumor perlecan into host blood vessels is likely mobilized by proteases easing the recruitment and diffusion of angiogenic growth factors into the tumor stroma (89). Gradients of perlecan expression have been observed in tumor vessels with the most reactive areas located at or around the sprouting edges, suggesting that tumor-derived perlecan can favor or induce the neovascularization of tumors (166, 190). Alternatively, host cells are proposed to synthesize perlecan as a defensive mechanism, with HS acting as a "sink" for growth factors by limiting their diffusion (154). The HS chains may be key elements that direct the intermolecular interactions that occur between perlecan and other BM components. The diverse substructure of HS chains might influence not only the growth factor-binding ability of perlecan but mediate roles in adhesion that can affect cancer cell proliferation and migration (86).

Tumor cells can also upregulate the production of several angiogenic factors such as FGF and VEGF in order to support their altered growth patterns and metabolism (154). For example, tumor vessels formed as a result of VEGF upregulation are

abnormal; these vessels are variably fenestrated and leaky, accompanied by a disorganized or loose BM (191) (**Figure 1**, Box 2). These conditions typically lead to high interstitial pressures, escalated tissue hypoxia and production of additional VEGF (192). Human prostate cancer cells, depleted of perlecan and grafted in mice, produced tumors of decreased size and vascularization, where the effects were correlated to reduced secretion of VEGF-A in the xenografts (193). The occurrence of hypoxia during the early stages of tumor growth has been shown to regulate a number of angiogenic growth factors and cytokines, including VEGF (194). The expression of regulatory enzymes responsible for HS chain synthesis is also subject to hypoxic influence with preferential synthesis of HS resulting in increased responsiveness of hypoxic endothelial cells to FGF-2 (195). The release of heparanase from tumor cells into the ECM promotes cleavage of HS fragments, which in turn liberates bound growth factors that act to further support tumor angiogenesis (196). Perlecan also plays a role in establishing cytokine gradients in the ECM which are utilized by cells to migrate through tissues, as in the case of angiogenesis (87, 197).

Invasion

Malignant tumors are characterized by their invasiveness into nearby tissues, followed by metastasis to distal locations away from the primary tumor site. In order for these processes to take place, a series of signaling mechanisms contribute to the breakdown of the surrounding ECM by activating or releasing various proteolytic enzymes. A key enzyme involved in HSPG processing is heparanase, which recognizes a HS sulfation motif to hydrolyze the glycosidic bond between glucuronic acid and glucosamine (198). Heparanase activity digests HSPGs, resulting in increased endothelial permeability that enables the passage of invading cells through established boundaries, and the release of sequestered growth factors and soluble HS fragments that support angiogenesis and tumor growth (196). It has also been proposed that reduced adhesion of tumor cells to the underlying ECM, as well as increased cell motility, is due to cleavage of cell surface HS by heparanase produced by the tumor cell itself (108). Notably, heparanase has also been recognized to participate in some non-enzymatic activities, separate from its involvement in ECM degradation and remodeling (199–201).

Upregulation of heparanase occurs in essentially all human tumors and is closely correlated with an invasive phenotype in experimental models and has been linked to worse outcomes in cancer patients (196, 202, 203). A few examples are presented. Lung metastatic melanoma cells overexpress heparanase isoform 1 (Hpa1) mRNA (up to 29-fold) compared to normal lung tissue (204). Hpa1 enzyme was identified around vascularized regions, as well as blood vessels near the invasion front in various representative models (204, 205). Heparanase over-expressing breast tumors are seven times larger and present significantly more vascularization (206). Friedmann et al. presented high levels of heparanase mRNA in lymph, liver, and lung tumor metastases with the highest amounts of both mRNA and enzyme detected in deeply invading colon carcinoma cells (207). Heparanase activity is upregulated in lung and brain cancers, with melanoma cells that are highly metastatic to the brain overexpressing Hpa1 (208, 209). Specimens from breast cancer patients showed that lymphocytes express heparanase and when serum collected from these patients was introduced to fresh lymphocytes, heparanase expression was stimulated in the normal lymphocytes (210). Furthermore, a non-metastatic cell type, transfected with the gene that encodes heparanase, acquired a metastatic phenotype (211). Hypoxia was found to augment heparanase activity and consequently invasion in ovarian cancer cell lines (212). Inversely, anti-sense targeting of heparanase weakens the invasive ability of carcinoma cells (213).

The importance of HSPG structure in tumor biology was demonstrated in a study where Liu et al. injected bacterial recombinant heparinase (Hep) I (which cleaves highly sulfated regions) and Hep III (which cleaves unsulfated regions) into melanoma challenged mice and found that the specificity of the enzymes dictated whether tumors regressed (Hep III) or advanced (Hep I) due to where the different enzymes cleaved HS (214). This finding demonstrated both the heterogeneity of HS and the fine control of biological function due to these different HS structures. They found that the resulting tumor cell GAG fragments were distinct following treatment with the different heparinase isoforms, with Hep III digestion causing up to 75% inhibition in tumor growth whereas fragments as a result of Hep I digestion significantly enhanced growth. Furthermore, the demonstrated effects were modulated by FGF-2 signaling, as Hep I-generated fragments promoted FGF-2 activity, whereas Hep III-generated fragments inhibited signaling, with additional implication of MAP kinase and FAK pathways. It should be noted that there is a difference in the mechanism by which mammalian-derived heparanase and bacterial-derived heparinase cleave HS; heparanase is a hydrolase, as opposed to heparinase which is an eliminase (215). In some instances, the overexpression of heparanase is linked to other enzyme activities. In addition to heparanase overexpression, melanoma cells were reported to exhibit 3-O-sulfotransferase gene hypermethylation and subsequent gene silencing (216). A study by Ma and Geng, showed that the cell adhesion molecule P-selectin, present on endothelial cells and activated platelets, was still capable of binding to a HS-like molecule displayed on melanoma cells despite the absence of its recognition motif (217). Interplay between a series of enzymes including 3-, 6-O-sulfotransferase and HSulf enzymes may transform HS to confer P-selectin binding ability and hence promote the migration of cells to secondary sites (81). Additionally, heparanase mediates upregulation of MMP-9, expressed from tumor cells, to indirectly stimulate invasion (218). In addition to the biological effects modulated by the HS chains of perlecan, perlecan-rich borders can resist cell invasion and serve as tissue boundaries (107). These borders include the glandular BM (219), the reactive stromal compartment (173), the vasculature (220), and bone marrow reticular matrix (221). Perlecan and MMP-7 co-localize at tissue boundaries when surveyed in prostate cancer sections, with MMP-7 proposed to act as a molecular switch by altering cancer cell behavior to favor cell dispersion and invasiveness (222, 223).

While increased expression of perlecan is shown in a number of tumor types (**Table 1**), its levels are also undetectable in other instances. Several early studies reported strong mRNA levels of

perlecan with the overexpressed perlecan protein deposited in the ECM and in tumor cells at the invading front (164, 171, 172, 224). These studies were supported by observations of inhibited tumor growth and angiogenesis (193, 225) or reduced cell proliferation and invasiveness (226) when perlecan was downregulated by anti-sense targeting. This is contrary to the findings reported by Mathiak et al. where anti-sense targeting of perlecan resulted in stimulation of tumor cell growth *in vitro* and *in vivo* accompanied with increased invasiveness in the ECM (227). It has been suggested that the lack of perlecan in these cases could perhaps be related to the tissue microenvironment preferentially favoring the diffusion of growth factors, which encourages tumor growth and metastasis (142, 154). Alternatively, Nerlich et al. reported high levels of perlecan mRNA in both tumor and stromal cells but then very low levels of perlecan protein present in tumor-associated BM (168, 169). Similarly, differences were observed between perlecan mRNA and secreted protein measured from stably transfected anti-sense perlecan targeting subclones, with reduction of >50% compared to the untransfected parental cell line (193). A recent study exploring the localization of perlecan in squamous cell carcinoma (SCC) reveals that perlecan and its binding growth factors namely VEGF [binds to HS chains (85)], Sonic Hedgehog (SHH) [HS and protein core (228)], and FGF-7 [protein core (147)] co-localize within the epithelial layer before invasion (176). Once the carcinoma cells started to invade, perlecan and FGF-7 were identified in the stromal space while VEGF and SHH remained at the epithelial layer. This correlates with other studies that suggested biosynthesis of perlecan was switched over from carcinoma cells to stromal cells (174, 190, 229, 230). The discrepancy between significantly enhanced mRNA synthesis and loss in protein deposition may also point to the activity of proteolytic enzymes or a post-translational block of protein synthesis or both (154).

Overexpression of perlecan in prostate cancer stroma has been linked to TNFα-mediated transcriptional induction (173). This suggests that perlecan transcription could be a part of cytokine-mediated innate immune response to cancer invasion. Perlecan has also been implicated in regulating prostate cancer progression via the SHH pathway (231). Franses and colleagues explored the role of endothelial cells in regulating cancer cell behavior, where perlecan silencing eliminated the ability of endothelial cells to suppress cancer invasiveness in both *in vitro* and *in vivo* models of breast and lung cancer (75). These findings indirectly contrast with the early work (discussed above) showing that perlecan depletion (albeit in cancer cells) slows tumor growth and reduces metastasis (193, 225, 226). The fact that perlecan acts in a cell context-specific manner could be a consideration for the contradicting data (142). It is important to note that perlecan derived from different cellular sources carries different HS structures and as such different growth factor binding and functional capabilities (94, 157). For example, Lord and colleagues have shown that the GAG chains differ between perlecan enriched from human coronary artery smooth muscle or endothelial cells and this influences their roles in mediating cell adhesion and proliferation, as well as FGF binding and signaling (157). Therefore, it can be summarized that tumor subtype, stage, degree of tumor differentiation, and/or various histological location and identifying reagent (i.e., primer region of interest or antibody epitope) may result in the different distribution of perlecan across the reported studies.

THERAPEUTIC TARGETING OF HEPARAN SULFATE PROTEOGLYCANS AND THEIR FUNCTION IN CANCER METASTASIS

Therapies that target HSPGs in cancer metastasis cover a range of modalities, highlighted in **Table 2**. Most therapies that target metastasis and the role of HS revolve around the inhibition of heparanase. The inhibition of heparanase eliminates the cleavage of HS chains and the release of bioactive molecules such as, FGFs, and VEGF, to disrupt the downstream events that are associated not only with the progression of cancer but also with cancer metastasis. Given the prevalence of cancer and the role of HSPGs in multiple events there is an extensive amount of literature, including a number of recent reviews (203, 247, 248), that detail the mechanisms of action of the range of therapeutics that are being developed. The following section will review the most recent advances in the field.

The first reports of heparanase inhibitors in an anti-cancer or anti-metastatic activity, stemmed from the use of heparin and low molecular weight heparins (LMWHs) (249). As heparin has a similar structure to HS, though a higher sulfated version, it competes with endogenous HS for both heparanase binding and substrate activity. However, the risk to patients regarding bleeding due to anticoagulant activity of heparin has limited their use as therapeutics for cancer and cancer metastasis, particularly as a long term therapeutic. Given the potential of both heparin and LMWHs, much effort has been directed toward either modifying or mimicking the structure heparin/LMWHs to remove the anticoagulant activity whilst retaining the ability to inhibit heparanase. The success of HS mimetics is clear through the number of these materials that have made it through to clinical trials. Modification of heparin through desulfation and glycol splitting has seen the development of roneparstat (250) and its investigation in a Phase I trial as a therapeutic for myeloma (232). In addition to roneparastat, HS mimetics muparfostat (PI-88) (233), neuparanib (N-402) (234), piixatimod (PG545) (251), have been, or are currently in clinical trials for use as a therapy targeting metastasis of melanoma or pancreatic cancer. More recent reports have detailed the use of these HS mimetics not only in the development of therapeutics, but the development of more representative models for testing anti-cancer/anti-metastatic therapeutics including patient-derived xenografts (237) and organoid models (238). Neuparanib has been shown to reduce tumor cell proliferation and invasion in an organoid model, and plasma levels of patients within a clinical trial cohort reported increased levels of tissue inhibitor of MMP-3 (238). The attempt at mimicking the structure of HS has seen the development of glycopolymers with well-defined sulfation patterns and the ability to optimize disaccharide length for peak heparanase inhibition (252), which reduced metastasis of breast cancer in a rodent model. The ability to design and synthesize HS mimicking structures that eliminate anti-coagulation activity

TABLE 2 | Summary of therapeutics that target heparan sulfate proteoglycans.

Therapeutic	Results or observations (specific compound reported in brackets)	References
HS mimetic/ heparanase inhibitor	In a Phase I clinical trial demonstrated safety though anti-myeloma efficacy was minimal (Roneparstat)	(232)
	Demonstrated safety in a Phase I clinical trial for melanoma [Muparfostat (PI-88)]	(233)
	Acceptable safety and encouraging signals of activity in patients with metastatic pancreatic cancer in Phase I clinical trial [Neuparanib (N-402)]	(234)
	Anti-metastatic effects in murine models of melanoma and lung cancer	(235)
	Inhibition of primary tumor growth and reduced metastasis in murine breast cancer model	(236)
	Acceptable safety and encouraging signals of activity in patients with metastatic pancreatic cancer in Phase I clinical trial	(234)
	Inhibition of metastasis from primary tumor in a lung cancer patient derived xenograft model	(237)
	Reduced MMP1 expression and increased TIMP3 expression in pancreatic cancer patients	(238)
LMWH	Reduced primary tumor and pulmonary metastasis in a murine melanoma model. LMWH was incorporated into a hydrogel system	(239)
Heparanase inhibitor	Benzoxazole derivatives demonstrated anti-metastatic potential via reduced expression levels of FGF-1, FGF-2, VEGF, and MMP-3 in a fibrosarcoma derived cell line	(240)
Sulfatase inhibitor	Inhibition of TGFβ1/SMAD and Hedgehog/GL1 pathways in hepatocellular carcinoma cell lines	(241)
	Reduced tumor size in mice implanted with xenograft pediatric glioblastomas	(242)
Immunotherapy	GPC-2 targeting antibody-drug conjugate reduced proliferation of GPC-2 expressing cells derived from neuroblastomas	(243)
	Monoclonal antibody that binds to GPC-3 demonstrated safety in a Phase I clinical trial for hepatocellular carcinoma	(244)
	GPC-3 CAR-T cells eliminated GPC-3 positive tumors in murine model of hepatocellular carcinoma.	(245, 246)

and target heparanase has more recently been facilitated with use of computational modeling to predict the anti-cancer/anti-metastatic potential (253–255).

In addition to the issues associated with anticoagulant activity, heparin also has a short half-life which can mean when administered intravenously that high dosages are required for a therapeutic effect or that there is the need for multiple injections. More recent reports have demonstrated the therapeutic use of heparin via incorporation or tethering to a substrate for targeted delivery. Reduction of metastasis in a lung cancer model was achieved with incorporation of heparin into a hydrogel system for local administration of the therapeutic (256). Tethering heparin to oligonucleotides via a cleavable linker that is pH sensitive (239), has also been demonstrated as a method of targeted delivery and the reduction of pulmonary metastasis in a melanoma model. Furthermore, delivery of LMWH, through tethering to micelles, reduced pulmonary metastasis in a breast cancer model, which was further reduced by using a delivery system that facilitated targeted co-delivery of the LMWH with the chemotherapy agent doxorubicin (257).

Despite their anti-metastatic properties, HS mimetics and polysaccharide derivatives have limitations due to their relatively high molecular weights, and rather heterogenous structures. More recently, there has been the exploration of small molecular inhibitors of heparanase, that overcome these limitations, for example benzimidazole and benzoxazole derivatives (258–260). Benzimidazole and benzoxazole derivates have been long studied in medicinal chemistry (261). Most recent advances in these derivatives include the synthesis of symmetrical analogs that demonstrated superior anti-heparanase activity as compared to non-symmetrical analogs (240), with the ability to not only inhibit heparanase, but also bind and sequester HS interacting growth factors and chemokines that modulate angiogenesis.

In addition to heparanase, sulfatases can modify HS via the removal of 6-*O*-sulfate groups and as such have been investigated as a targeting molecule. The compound designated OK-007, 2,4-disulfophenyl-*N*-tert-butylnitrone, inhibits the enzymatic activity of Sulf2. This compound was initially explored as a treatment for acute ischemic stroke (262), though has since been investigated as a potential therapeutic for HCC (241) and glioblastoma (242). Coutinho de Souza et al. (242) demonstrated the ability for OKN-007 to reduce cell proliferation and the expression of the receptor for platelet derived growth factor, and the authors speculated potential anti-angiogenic properties of OKN-007.

More recently, monoclonal antibody therapy, a form of immunotherapy, has been explored as a route to target HSPGs. Though, these therapies have been mainly focused toward targeting primary rather than secondary tumors. Monoclonal antibodies targeting GPC-2 have been developed as a therapeutic for neuroblastoma (243), and antibodies targeting GPC-3 have progressed to phase I trials in HCC (244). More recently GPC-3 in HCC has been used as a target in chimeric antigen receptor, or CAR T-cell therapy (245, 246, 263), with the therapy demonstrating the ability to reduced HCC tumors in a xenograft model (245).

CONCLUSIONS

The role of HSPGs in cancer metastasis is through the interaction of the HS chains or PG protein core with key biological molecules associated with metastatic events. The non-templated heterogeneous structure of HS modulates these specific interactions between mediators, influencing events in the metastatic cascade. Furthermore, the increase in heparanase expression in multiple cancer types results in the cleavage of HS chains and release of mediators involved in these events. HSPGs, including perlecan, have antithetic roles in

cancer and metastasis through the interaction with biological molecules. The subtle differences in HSPG structure, particularly that of HS, results in a family of molecules that behave as both pro- or anti-metastatic factors. Thus, due to the structure specific interactions between HS and mediators of metastatic events, future therapeutics that target HSPGs and their cleaving enzymes need to target specific HS or heparanase binding structures, and ideally have targeted delivery, to ensure both efficacy and reduced off-target effects to truly improve patient outcomes.

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Heparanase Loosens E-Cadherin-Mediated Cell-Cell Contact via Activation of Src

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Activity of heparanase, responsible for cleavage of heparan sulfate (HS), is strongly implicated in tumor metastasis. This is due primarily to remodeling of the extracellular matrix (ECM) that becomes more prone to invasion by metastatic tumor cells. In addition, heparanase promotes the development of blood and lymph vessels that mobilize disseminated cells to distant organs. Here, we provide evidence for an additional mechanism by which heparanase affects cell motility, namely the destruction of E-cadherin based adherent junctions (AJ). We found that overexpression of heparanase or its exogenous addition results in reduced E-cadherin levels in the cell membrane. This was associated with a substantial increase in the phosphorylation levels of E-cadherin, β-catenin, and p120-catenin, the latter recognized as a substrate of Src. Indeed, we found that Src phosphorylation is increased in heparanase overexpressing cells, associating with a marked decrease in the interaction of E-cadherin with β-catenin, which is instrumental for AJ integrity and cell-cell adhesion. Notably, the association of E-cadherin with β-catenin in heparanase overexpressing cells was restored by Src inhibitor, along with reduced cell migration. These results imply that heparanase promotes tumor metastasis by virtue of its enzymatic activity responsible for remodeling of the ECM, and by signaling aspects that result in Src-mediated phosphorylation of E-cadherin/catenins and loosening of cell-cell contacts that are required for maintaining the integrity of epithelial sheets.

Keywords: heparanase, E-cadherin, Src, phosphorylation, cell migration

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INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) consist of a protein core to which several linear heparan sulfate (HS) chains are covalently linked to specific serine residues. HSPGs bind to and assemble extracellular matrix (ECM) proteins (i.e., laminin, fibronectin, collagen type IV) and thereby contribute significantly to the physical (insolubility) and biological properties of the ECM (1–6). In addition, transmembrane (syndecans) and phospholipid-anchored (glypicans) HSPGs have a co-receptor role in which the proteoglycan, in concert with other cell surface molecules, comprises a functional receptor complex that facilitates signal transduction (1–3). The ECM provides an essential physical barrier between cells and tissues, plays an important role in cell growth, migration, differentiation and survival (7), and undergoes continuous remodeling during development and in certain pathological conditions such as wound healing and cancer (7, 8). ECM remodeling enzymes are thus expected to have a profound effect in many biological settings.

Heparanase is an endo-β-D-glucuronidase capable of cleaving HS side chains at a limited number of sites (9, 10). Heparanase activity is strongly implicated in tumor metastasis, a consequence of remodeling the ECM underlying epithelial cells (9–11). Similarly, heparanase activity was found to promote the motility of vascular endothelial cells and activated cells of the immune system (12–16). HS also bind a multitude of growth factors, chemokines, cytokines, and enzymes, thereby functioning as a low-affinity storage depot (17). Cleavage of HS side chains by heparanase is therefore expected not only to alter the integrity of the ECM but also to release HS-bound biological mediators that can function locally in a highly regulated manner. Intense research effort in the last two decades revealed that heparanase expression is often increased in human tumors (18, 19). In many cases, heparanase levels correlate with increased tumor metastasis, vascular density, and shorter postoperative survival of cancer patients (14, 16, 18, 20), thus providing strong clinical support for the pro-tumorigenic function of the enzyme and encouraging the development of heparanase inhibitors as anti-cancer drugs (21, 22). The pro-metastatic function of heparanase is attributed primarily to the cleavage of HS and remodeling of the ECM. In addition, heparanase promotes tumor vascularization (blood and lymph vessels) that mobilize disseminating cells to distant organs. Here, we show that heparanase disrupts adherent junctions (AJ) by augmenting the phosphorylation of E-cadherin and catenin family members (β-catenin, p120-catenin) that play an instrumental role in epithelial sheet adhesion, integrity, and function. This is mediated via increased Src phosphorylation in response to heparanase because treatment of heparanase overexpressing cells with Src inhibitors restored AJ, resulting in decreased cell migration. These results reveal another mechanism utilized by heparanase to promote cell dissemination and tumor metastasis.

MATERIALS AND METHODS

Antibodies and Reagents

Anti E-cadherin (sc-8426), anti β-catenin (sc-7199), anti-paxillin (sc-5574), anti Src (sc-18 and sc-19), and anti-phosphotyrosine (sc-7020) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Polyclonal antibody to phospho-Src (Tyr416) was purchased from Cell Signaling (Beverly, MA). Anti-actin and anti-ɤ-catenin (plakoglobin) antibodies were purchased from Sigma (St. Louis, MO). Anti p120-catenin was purchased from Becton Dickinson (Mountain View, CA); Anti heparanase polyclonal antibody (#1453) has been described previously (23). The selective Src (PP2) and EGFR (CL-387,785) inhibitors were purchased from Calbiochem (San Diego, CA) and were dissolved in DMSO as stock solutions. DMSO was added to the cell culture as control. Phalloidin-TRITC and streptavidin-HRP were purchased from Sigma.

Cell Culture and Transfection

FaDu pharynx carcinoma cells were kindly provided by Dr. Eben L. Rosenthal (the University of Alabama at Birmingham, Birmingham, AL) (24); JSQ3 nasal vestibule carcinoma cells were kindly provided by Dr. Ralph Weichselbaum (University of Chicago, Chicago, IL) (25); SIHN-013 laryngeal carcinoma cells were kindly provided by Dr. Sue Eccles (Institute of Cancer Research, Sutton, Surrey, UK) (26); T47D breast carcinoma cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's (DMEM) or RPMI medium (T47D) supplemented with glutamine, pyruvate, antibiotics and 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37°C. For stable transfection, cells were transfected with heparanase gene constructs using the FuGene reagent according to the manufacturer's (Roche) instructions, selected with Zeocin (Invitrogen, Carlsbad, CA) for 2 weeks, expanded and pooled, as described (27, 28). Cells were passed in culture for no more than 3 months after being thawed from authentic stocks.

HEK 293 cells, stably transfected with the human heparanase gene construct in the mammalian pSecTag vector (Invitrogen), were kindly provided by ImClone Systems (New York, NY). The cells were grown in DMEM supplemented with 10% FCS, glutamine, pyruvate, and antibiotics. For heparanase purification, the cells were grown overnight in serum-free-DMEM and the conditioned medium (∼1 liter) was purified on a Fractogel EMD $SO3^-$ (MERCK) column. The bound material was eluted with 1 M NaCl and was further purified by affinity chromatography on anti-c-Myc (Santa Cruz Biotechnology) column. We obtained at least 95% pure heparanase preparation by this two-step procedure (29).

Cell Fractionation, Immunoprecipitation, and Protein Blottin

Isolation of plasma membrane fraction was carried out essentially as described (30). Briefly, T47D cells (3×10^8) were harvested by EDTA (2.5 mM), washed twice with PBS, suspended in 1 ml extraction buffer (10 mM Tris/acetic acid buffer, pH 7.0, supplemented with 250 mM sucrose) and were incubated for 20 min on ice. Cells were then homogenized in 5 ml Potter-Elvehjen homogenizer followed by centrifugugation at 2,000 × g for 2 min; The supernatant was collected and centrifuged at 4,000 x g for 2 min to pellet a fraction enriched with plasma membranes. Membrane proteins were dissolved with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 1 mM orthovanadate, 1 mM PMSF) and equal amounts of protein were subjected to immunoblotting.

Preparation of cell lysates, immunoprecipitation, and immunoblotting was performed essentially as described (27, 28). Briefly, cell cultures were pretreated with 1 mM orthovanadate for 10 min at 37°C, washed twice with ice-cold PBS containing 1 mM orthovanadate and scraped into lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM orthovanadate, 1 mM PMSF) containing a cocktail of proteinase inhibitors (Roche). Total cellular protein concentration was determined by the BCA assay according to the manufacturer's instructions (Pierce, Rockford, IL). Thirty μg of cellular protein were resolved on SDS polyacrylamide gel, and immunoblotting was performed, as described (23, 29). Immunoblots were subjected to densitometry analyses and the relative intensity of bands (i.e., fold change) is presented underneath the gel. Changes in protein

phosphorylation is presented in comparison to control (Vo) cells, set arbitrarily to a value of 1, and following normalization to the total levels of the protein in the cell lysate. Immunoprecipitation (IP) was carried out essentially as described (31). Briefly, 600 μg of cellular protein were brought to a volume of 1 ml in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% NP-40, incubated with the appropriate antibody for 4 h on ice followed by incubation with protein G-Sepharose (Rosche; 60 min on ice). Beads were washed twice with the same buffer supplemented with 5% sucrose. Sample buffer was added, and samples were boiled and subjected to gel electrophoresis and immunoblotting, as described above.

Surface Biotinylation

Surface biotinylation was carried out by using EZ link Sulfo-NHS-SS-Biotin according to the manufacture's (Thermo Fisher Scientific) instructions. Briefly, Sulfo-NHS-SS-Biotin was dissolved in PBS containing Ca++ and Mg++ to a concentration of 0.5 mg/ml and added to cell culture for 30 min on ice. Cell culture was then washed (×3) with ice-cold quenching solution (50 mM glycine in PBS containing Ca++ and Mg++). Cell lysates were then prepared and subjected to IP for E-cadherin, followed by immunoblotting with streptavidin-HRP (Sigma).

Immunocytochemistry

Immunofluorescent staining was performed essentially as described (23, 27, 32). Briefly, cells were grown on glass coverslips for 18 h. Heparanase (1 μg/ml) was then added for the time indicated, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 20 min. Cells were then permeabilized for 1 min with 0.5% Triton X-100, washed with PBS and incubated in PBS containing 10% normal goat serum for 1 h at room temperature, followed by 2 h incubation with the indicated primary antibody. Cells were then extensively washed with PBS and incubated with the relevant Cy2/Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h, washed and mounted (Vectashield, Vector, Burlingame, CA). Wound healing migration assay was carried out essentially as described (29).

Flow Cytometry

Cells were detached with 2.5 mM EDTA, centrifuged at 1000 RPM for 4 min., washed with PBS and counted. Cells (2×10^5) were centrifuged and the pellet was then resuspended in PBS containing 1% FCS and incubated with FITC conjugated anti-E-cadherin antibody for 40 min on ice. Cells were then washed twice with PBS and analyzed using a FACSCalibur fluorescent activated cell sorter and CellQuest software (Becton Dickinson, Mountain View, CA), as described (29).

Statistics

Results are shown as means ±SE. GraphPad Instat software was used for statistical analysis. The differences between the control and treatment groups were determined by two-tailed Student's *t*-Test. Statistically significance is presented according to the common use of $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Heparanase Disrupts Adherent Junctions (AJ)

Heparanase expression is often induced in carcinomas and is associated with increased tumor metastasis and bad prognosis (19, 33), but the effect of heparanase on AJ has not been reported yet. We noticed that overexpression of heparanase in T47D breast carcinoma cells resulted in more dispersed cell colonies (**Figure 1A**, left). These cells also exhibited more abundant focal contacts evident by paxillin staining (**Figure 1A**, right), typical of migrating cells. A similar increase in paxillin staining was observed following exogenous addition of latent heparanase (65 kDa) to SIHN-013 laryngeal and JSQ3 nasal vestibule carcinoma cells (**Supplementary Figure 1A**). Notably, overexpression of heparanase was associated with decreased E-cadherin at cell-cell borders evident by immunofluorescent staining (**Figure 1B**), cell surface biotinylation (**Supplementary Figure 1B**, upper panel), and immunoblotting of cell membrane fractions (**Supplementary Figure 1B**, lower panel). Moreover, overexpression of heparanase was associated with a decreased interaction (3-fold) of E-cadherin with β- and ɤ-catenin (**Figure 1C**) which is essential to connect E-cadherin with the actin cytoskeleton and establish functional AJ. Increased migration of cells out of well-organized colonies was observed following exogenous addition of latent heparanase protein (**Figure 1D**) and is best demonstrated by time-lapse microscopy (**Supplementary Videos 1**, **2**). Reduced levels of β-, ɤ-, and p120-catenin at cell-cell borders were evident already 30 min after the addition of heparanase, and the catenins that were retained on the cell surface appeared discontinued and were arranged in a patchy manner (**Figure 1E**, **Supplementary Figure 1C**, left and middle panels). The rapid decrease of E-cadherin/catenins from cell-cell borders may suggest the involvement of a signaling pathway elicited by heparanase.

Disruption of AJ by Heparanase Is Mediated by Src

We have reported previously that overexpression of heparanase augments the phosphorylation levels of p120-catenin (34), a catenin-family member originally identified as a Src substrate (35). Indeed, overexpression of heparanase in T47D cells (**Figure 2A**, upper panel) was associated with increased phosphorylation levels of Src (3.3-fold; **Figure 2A**, second panel) and p120-catenin (3.6-fold; **Figure 2A**, fourth panel), in agreement with earlier reports showing that heparanase enhances Src phosphorylation (27, 34, 36). Similarly, the phosphorylation levels of E-cadherin and β-catenin were also augmented substantially in cells overexpressing heparanase (2.8- and 4.2-fold, respectively; **Figure 2A**, sixth and eighth panels). Given that E-cadherin/catenins phosphorylation results

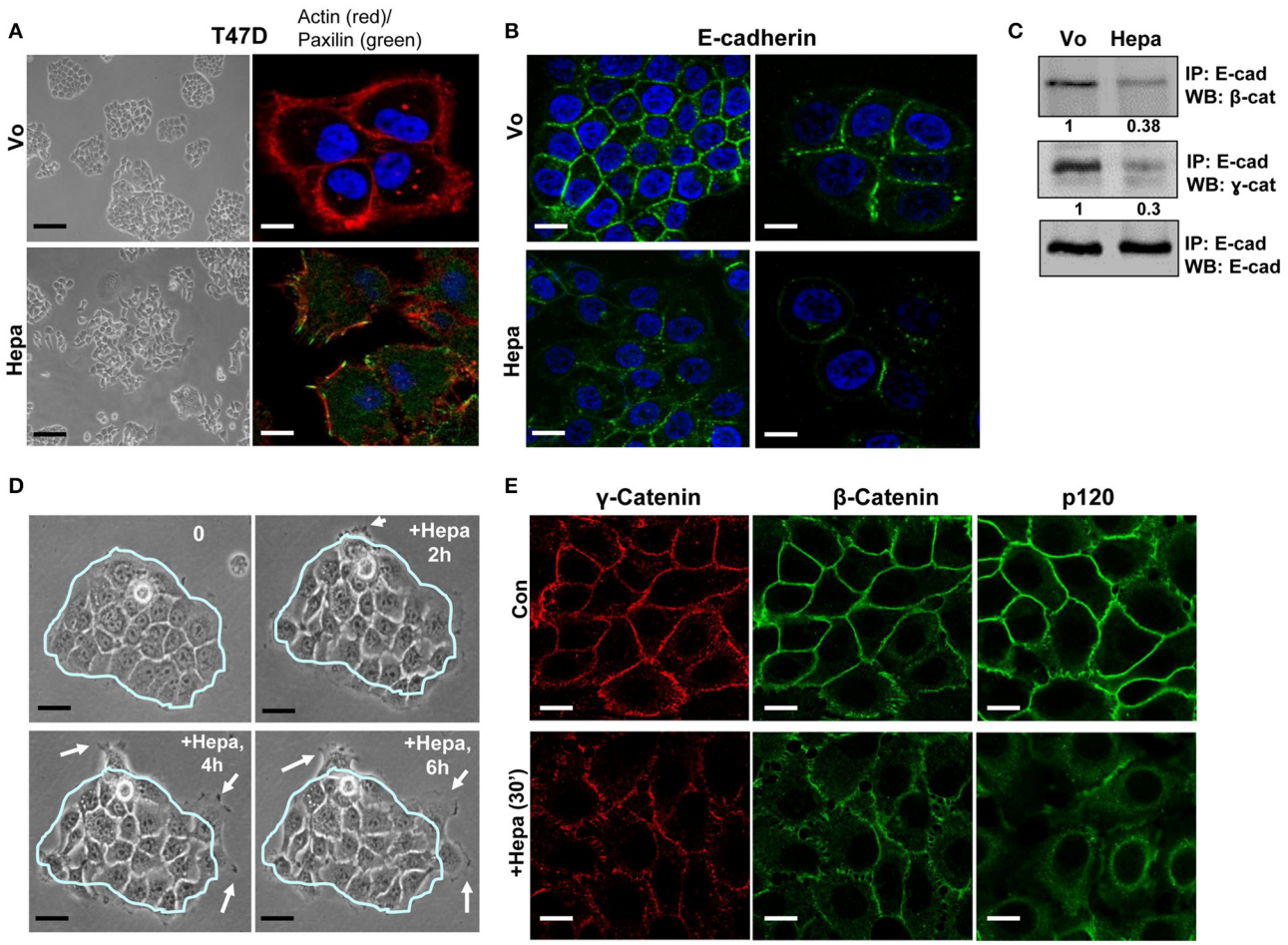

FIGURE 1 | Heparanase affects cell-cell contacts and disrupts AJ. **(A)** Dispersed cell colonies. T47D breast carcinoma cells were transfected with an empty vector (Vo) or heparanase gene construct (Hepa), and their growth pattern was examined. Shown are representative cell cultures. Note that while control (Vo) cells grew in typical well-organized colonies, heparanase overexpressing cells show dispersed cell colonies (left panels). Control (Vo) and heparanase overexpressing cells were fixed with 4% PFA and following permeabilization were stained with phalloidin-TRITC to label the actin cytoskeleton (red), and paxillin (green) that typically labels adherent junctions (right panels). Note abundant paxillin staining in heparanase overexpressing cells. Scale bars represent 60 (left panels) and 10 microns (right panels). **(B)** Decreased E-cadherin staining in heparanase cells. Control (Vo) and heparanase overexpressing cells (Hepa) were subjected to immunofluorescent staining applying anti-E-cadherin antibody. Shown are representative images (confocal microscopy) at ×100 (left) and ×200 (right) magnifications merged with nuclear labeling (DAPI; blue). Note decreased E-cadherin at cell-cell borders upon heparanase overexpression. Scale bars represent 20 (left panels) and 10 (right panels) microns. **(C)** Immunoprecipitation. Lysates of control (Vo) and heparanase overexpressing cells (Hepa) were subjected to IP applying anti-E-cadherin antibody, followed by immunoblotting with anti-β-catenin (upper panel), ɤ-catenin (second panel), and anti-E-cadherin (lower panel) antibodies. Densitometry analysis of protein band intensity is shown below each panel in relation to its level in control (Vo) cells, set arbitrarily to a value of 1. Note decreased association of E-cadherin with catenins in heparanase cells. **(D)** Exogenous addition. T47D cells were seeded at low density, and cell colonies were allowed to form. Colonies were then photographed and their morphology was inspected over time following treatment with latent heparanase added exogenously (1 μg/ml) to the cell culture medium. Shown is a typical colony before (0) and after the addition of heparanase for 2, 4, and 6 h. Note that cells are migrating out of the colony (white arrows) after the addition of heparanase. Scale bars represent 30 microns. **(E)** Immunofluorescent staining. T47D cells were left untreated (Con) or were treated for 30 min with latent heparanase (1 μg/ml) added exogenously to the cell culture medium. Cells were then fixed, permeabilized, and subjected to immunofluorescent staining applying anti-ɤ-catenin (left panels), anti-β-catenin (middle panels), and anti-p120-catenin (right panels) antibodies. Note decreased and less organized staining of the catenins following the addition of heparanase. Scale bars represent 10 microns.

in the dissociation of AJ (37, 38), we investigated whether Src inhibitors (e.g., PP2) would restore AJ integrity in cells overexpressing heparanase. To this end, control (Vo) and heparanase (Hepa) cells were treated with DMSO as vehicle control (Con) or with PP2, and cell extracts were subjected to IP for E-cadherin. While the total levels of E-cadherin appeared similar in control (Vo) and heparanase (Hepa; **Figure 2B**, upper panel) cells, its association with β-catenin was strikingly lower in heparanase overexpressing cells (Hepa; **Figure 2B**, second panel, Con), but was increased prominently in heparanase cells treated with PP2 (PP2; **Figure 2B**, second panel). Likewise, PP2 treatment was associated with a marked decrease in the phosphorylation levels of E-cadherin (PP2; **Figure 2B**, third panel), β-catenin (PP2; **Figure 2B**, fifth panel), p120-catenin

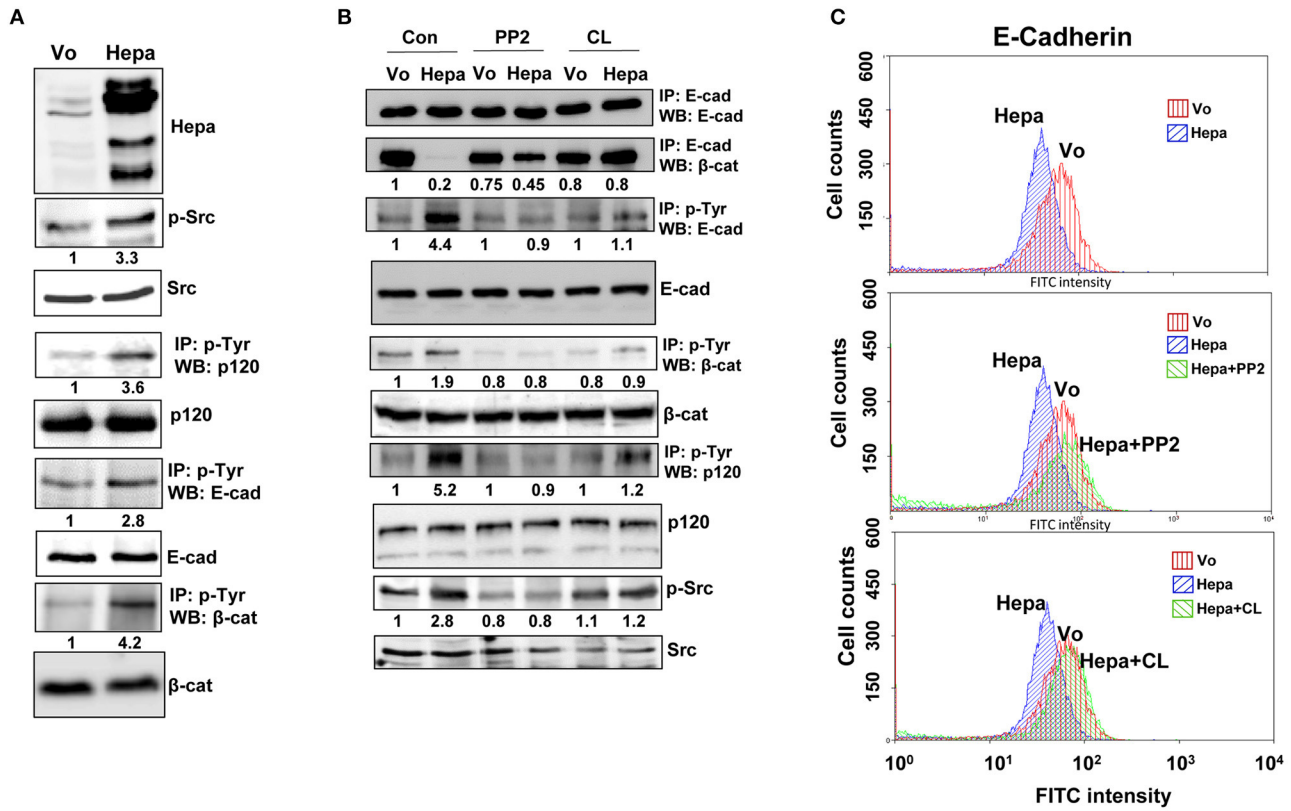

FIGURE 2 | Heparanase enhances the phosphorylation of E-cadherin and catenins via activation of Src. **(A)** immunoblotting. Lysates of control (Vo) and heparanase (Hepa) overexpressing cells were subjected to immunoblotting applying anti-heparanase (upper panel), anti-phospho–Src (p-Src; second panel), and anti-Src (third panel) antibodies. Cell lysates were subjected to IP with anti-phospho-tyrosine antibody (p-Tyr), followed by immunoblotting with anti-p120-catenin (fourth panel), anti-E-cadherin (sixth panel), and anti-β-catenin (eighth panel) antibodies. Densitometry analysis of protein band intensity is shown below each panel in relation to its level in control (Vo) cells, set arbitrarily to a value of 1, and following normalization to the total levels of Src, p120, E-Cadherin and β-catenin (third, fifth, seventh, and ninth panels, respectively) in the cell lysates. **(B)** Inhibitors of Src and EGFR restore the association of E-cadherin with β-catenin. Control (Vo) and heparanase overexpressing cells (Hepa) were treated with vehicle (DMSO) as control (Con) or with inhibitors of Src (PP2; 5 μM) or EGFR (CL-387,785; 0.01 μM) for 3 h. Cell lysates were then prepared and subjected to IP with anti-E-cadherin antibody, followed by immunoblotting with anti-E-cadherin (upper panel) and anti-β-catenin (second panel) antibodies. Lysates were similarly subjected to IP with anti-phosphotyrosine (p-Tyr) antibody, followed by immunoblotting with anti-E-cadherin (third panel), anti-β-catenin (fifth panel), and anti-p-120-catenin (seventh panel) antibodies. Cell lysates were similarly immunoblotted applying anti-phospho-Src (p-Src; ninth panel) and anti-Src (lower panel) antibodies. Densitometry analysis of protein band intensity is shown below each panel in relation to its level in control (Vo) cells, set arbitrarily to a value of 1, and following normalization to the total levels of E-Cadherin, β-catenin, p120, and Src (fourth, sixth, eighth, and tenth panels, respectively) in the cell lysates. Corresponding control (Vo) and Hepa cells treated with DMSO (vehicle), PP2, or CL-387,785 were detached with EDTA and subjected to FACS analyses applying anti-E-cadherin antibody **(C)**. Note that inhibition of Src or EGFR restores the association of E-cadherin with β-catenin in Hepa cells.

(PP2; **Figure 2B**, seventh panel), and Src (**Figure 2B**, ninth panel). Interestingly, similar results were obtained in cells treated with an inhibitor of the EGF receptor (EGFR), CL-387,785 (**Figure 2B**, CL). This may suggest that Src phosphorylates and activates the EGFR (27), leading to the disruption of AJ (39).

In order to further reveal the restoration of AJ by Src inhibitor evident by co-IP (**Figure 2B**), we subjected control and PP2 treated cells to FACS analyses. While the levels of E-cadherin at the cell surface was decreased in cells overexpressing heparanase vs. control (Vo) cells (**Figure 2C**, upper panel), in agreement with the surface biotinylation and membrane fractionation approaches (**Supplementary Figure 1B**), treatment with PP2 (**Figure 2C**, second panel) and CL-387,785 (**Figure 2C**, lower panel) restored its localization at the cell surface to the levels of control (Vo) cells. This was further evident by immunofluorescent staining (**Figure 3**), clearly depicting that treatment of heparanase overexpressing cells with PP2 results in recruitment of E-cadherin to the cell surface and restoration of AJ.

Heparanase Promotes Cell Migration via Activation of Src

Cell-cell contact and AJ integrity play an instrumental role in cell migration. To examine the consequences of increased E-cadherin/catenins phosphorylation in heparanase overexpressing cells and the associated disruption of AJ on cell migration, we employed a wound-healing assay. We found that heparanase cells migrate faster than control (Vo) cells. This was evident already 24 h post wounding (Control; **Figures 4A,B**,

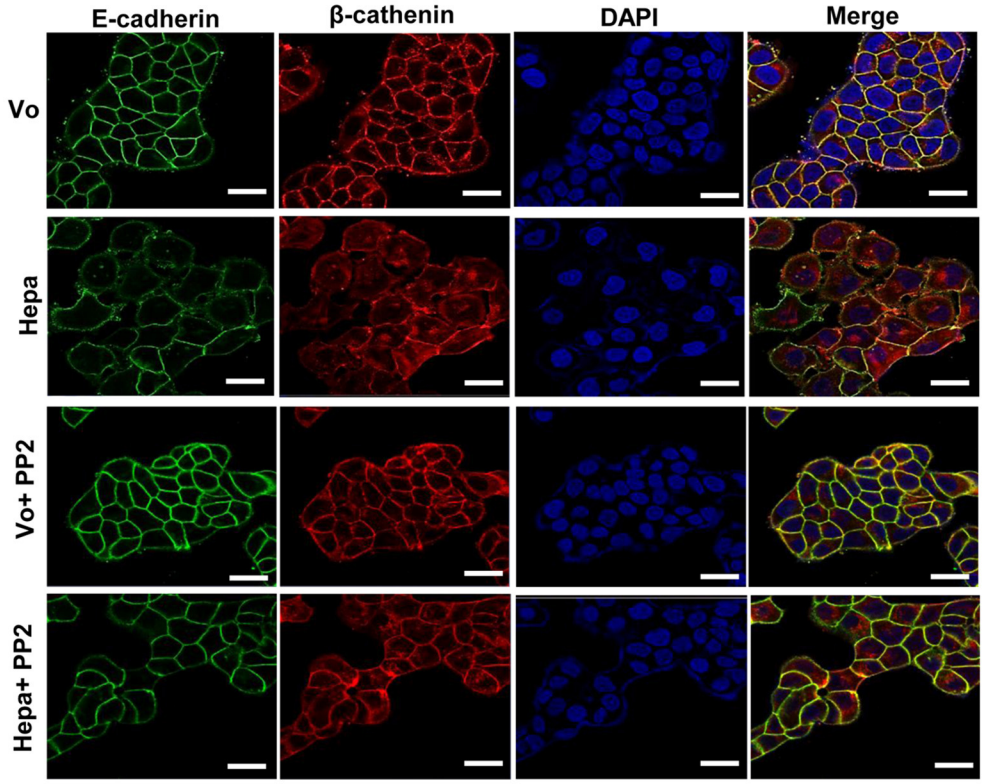

FIGURE 3 | Immunofluorescent staining. Control (Vo) and heparanase overexpressing T47D cells (Hepa) were left untreated or were treated with PP2 (5 μM) for 3 h. Cells were then fixed with 4% PFA, permeabilized, and subjected to immunofluorescent staining applying anti-E-cadherin (green) and anti-β-catenin (red) antibodies. Merged images are shown in the right panels together with nuclear counterstaining (blue). Shown are representative images (confocal microscopy) at ×100 magnification. Note that far more E-cadherin and β-catenin are recruited to cell-cell contacts following Src inhibition with PP2. Scale bars represent 15 microns.

24 h; $p < 0.05$ for Vo vs. Hepa), and became most evident by 48 h when heparanase cells filled the wounded area almost completely (Control; **Figure 4A**, lower panels & **Figure 4B**; $p < 0.01$ for Vo vs. Hepa). Importantly, the pro-migratory function of heparanase was abrogated by inhibitors of Src (PP2; **Figures 4A,B**; $p < 0.001$ for Hepa vs. Hepa+PP2 at 24 and 48 h) and EGFR (CL; **Figures 4A,B**; $p < 0.05$ and $p < 0.001$ for Hepa vs. Hepa+CL at 24 and 48 h, respectively), along with the restoration of AJ (**Figures 2B,C**, **3**), further signifying that heparanase promotes cell migration by activation of Src, leading to disruption of E-cadherin-based cell-cell contact.

DISCUSSION

Heparanase has long been implicated in tumor metastasis. This notion is now well-accepted and supported by compelling pre-clinical and clinical data (19, 20, 33, 40). The pro-metastatic function of heparanase is largely attributed to its enzymatic activity capable of cleaving HS and, consequently, remodeling of the ECM underlying epithelial and endothelial cells. In addition, heparanase enhances the formation of new blood and lymph vessels (19, 20, 28, 33, 40), thereby promoting the mobilization of disseminating tumor cells to distant organs. Here, we describe a new mechanism by which heparanase can promote cell dissemination namely, disruption of AJ. E-cadherin-based AJ are characteristic of all epithelial cells. Through the homophilic association of E-cadherin molecules expressed on neighboring cells, they ensure intercellular adhesion between epithelial cells and regulate many key aspects of epithelial biology (37). AJ structures are stabilized by the accumulation of a dense actin filaments-based network, mediated by anchoring E-cadherin clusters to the inner cytoskeleton. The link to the actin cytoskeleton is mainly mediated by β-catenin via its association with α-catenin (37, 38). In mammalian cells, the E-cadherin/catenin complex and AJ stability are tightly regulated by phosphorylation, where Src kinase and Src-family members are thought to play an instrumental role (37–39). More specifically, phosphorylation of β-catenin by Src results in reduced association with E-cadherin and α-catenin, leading to AJ disruption and subsequent decreased cell-cell adhesion (37–39). Importantly, such a decrease in cell-cell contacts and loss of E-cadherin has been associated with advanced tumor stages and poor prognosis in patients with cancer (38, 41).

Previously, we have reported that heparanase enhances the phosphorylation of Src, associating with increased cell proliferation and colony formation in soft agar (27, 34, 36). The mechanism by which heparanase enhances the phosphorylation

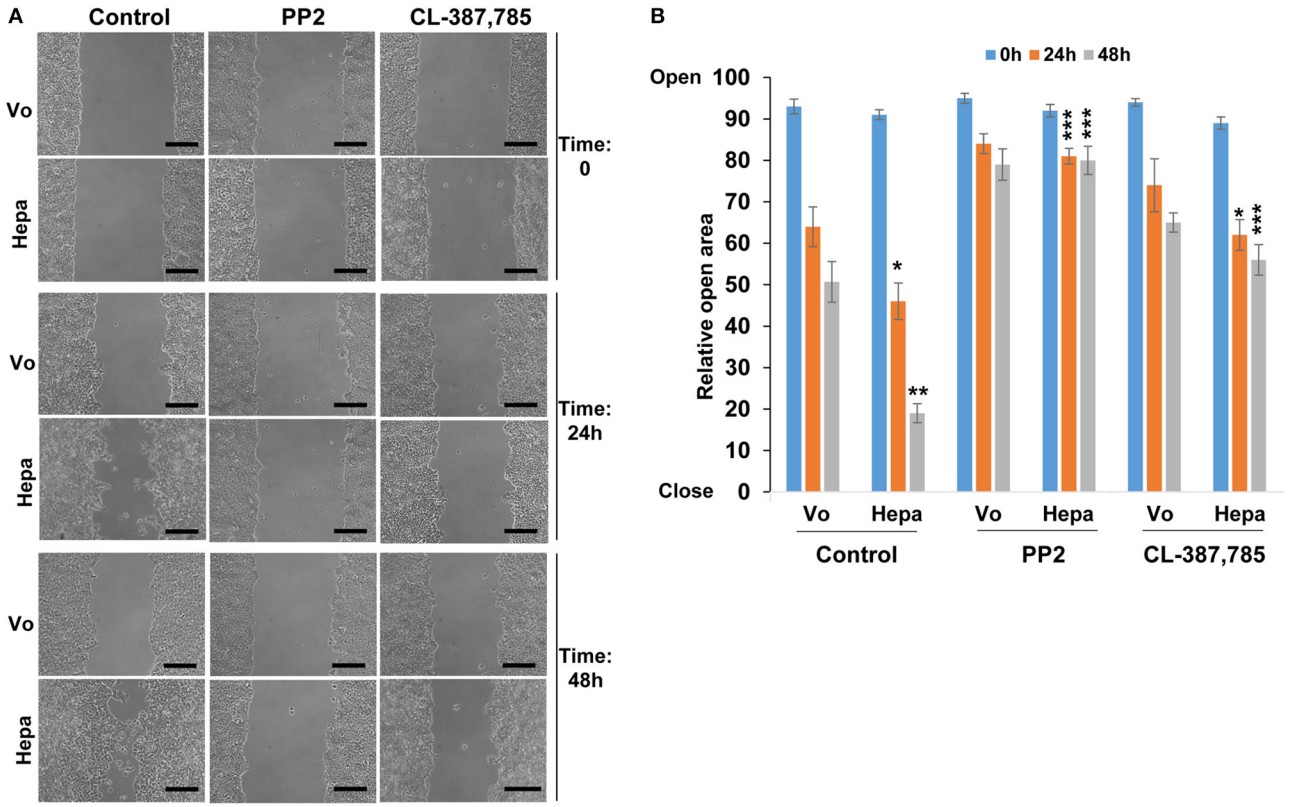

FIGURE 4 | Cell migration. Control (Vo) and heparanase overexpressing (Hepa) T47D cells were grown to confluence. Cultures were then scratched and were treated with DMSO (vehicle) as control or with PP2 (5 μM) or CL-387,785 (0.01 μM). Cell migration into the wounded area was observed over 2 days. Shown are representative images taken immediately after wounding (Time 0), and 24 and 48 h thereafter **(A)**. Quantification of relative wound closure is shown graphically in **(B)**. Note that heparanase overexpressing cells fill the wounded area faster than control cells and this increase in cell migration is reversed by Src- and to a lesser extent by EGFR- inhibitor. Scale bars represent 150 microns.

of Src is not entirely clear, but seems to be independent of heparanase enzymatic activity. This was concluded because increased Src phosphorylation was observed in cells over expressing heparanase that was mutated in glutamic acids 225 and 343 that comprise the enzyme active site (42), or heparanase that was deleted for the heparin binding domain [amino acids 270–280; Δ10; (31)] (27, 36), indicating that Src activation does not require heparanase enzymatic activity or its interaction with HS. Thus, inhibitors of heparanase activity such as HS-mimetics or JG6, a marine-derived oligosaccharide (43, 44), are not expected to attenuate this function of heparanase. It is possible, nonetheless, that Src activation is downstream to the activation of the epidermal growth factor receptor (EGFR) (27), focal adhesion kinase (FAK) (44), or integrin (29) by heparanase. Activation of Src family members such as Fyn, Lyn, or Hck by heparanase has not been so far reported. Here, we confirm and further expand the consequences of Src activation by heparanase. Notably, overexpression of heparanase in T47D cells was associated with increased Src phosphorylation, more dispersed cell colonies (**Figure 1A**, **Supplementary Videos 1**, **2**), and decreased E-cadherin at cell-cell borders. This was evident by immunofluorescent staining (**Figure 1B**), FACS analyses (**Figure 2C**), surface biotinylation (**Supplementary Figure 1B**, upper panel), and immunoblotting of membrane fractions (**Supplementary Figure 1B**, lower panel). Moreover, the phosphorylation levels of E-cadherin, p120-catenin, and β-catenin were increased markedly in cells overexpressing heparanase (**Figures 2A,B**), modifications that are highly associated with disruption of AJ (38, 41). Indeed, IP experiments revealed a remarkable decrease in the association of E-cadherin with β-catenin (**Figure 2B**), which was restored in heparanase cells treated with Src inhibitor (PP2; **Figure 2B**). Similarly, localization of E-cadherin to the cell membrane, evident by FACS analyses and immunofluorescent staining, was increased in heparanase cells treated with PP2 (**Figures 2C**, **3**). Disruption of AJ typically leads to reduced cell-cell contacts and increased cell migration. Indeed, heparanase was noted to promote cell adhesion and cell migration in a manner that seems not to involve its enzymatic activity (29, 32, 45, 46). Our results suggest that increased cell migration by heparanase involves Src-mediated phosphorylation of E-cadherin/catenins. This notion is supported by the observed increased cell migration and wound closure of T47D cells overexpressing heparanase, and decreased wound closure following treatment with PP2

(**Figure 4**). Importantly, treatment of mice with PP2 for 3 weeks markedly reduced the rate of liver metastasis by colon carcinoma cells (47), thus signifying the critical role of Src in disrupting AJ integrity, leading to cell dissemination and tumor metastasis. Reduced E-cadherin expression is often observed in the context of epithelial-mesenchymal transition (EMT), accompanied by increased levels of mesenchymal proteins such as N-cadherin, vimentin, and fibronectin (48). We did not observe changes in the expression levels of E-cadherin upon heparanase overexpression nor activation of an EMT program (i.e., induction of Twist, Snail, Slug, or ZEB transcription factors) (data not shown), suggesting that Src activation is the main force that drives E-cadherin/catenin phosphorylation and disruption of AJ. Notably, heparanase was found to elicit EMT in the context of kidney injury (49–52), suggesting that activation of the EMT program by heparanase can occur, depending on the biological context and experimental system employed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

IV and NI designed the research. VC-K performed the research. VC-K, IV, and NI analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00002/full#supplementary-material

Supplementary Figure 1 | (A) Paxillin staining. Latent heparanase (1 μg/ml) was added exogenously to SIHN-013 laryngeal carcinoma (013; left) and JSQ3 nasal vestibule carcinoma (right) cells. After 2 h, cells were fixed with 4% PFA, permeabilized, and subjected to immunofluorescent staining applying anti-paxillin antibody (green) along with phalloidin-TRITC (red) staining. Note increased paxillin staining at focal contacts following the addition of heparanase. Scale bars represent 10 microns. **(B)** Localization of E-cadherin on the cell membrane is decreased in heparanase overexpressing cells. T47D cells were subjected to surface biotinylation as described under "Materials and Methods." Cell extracts were then prepared and subjected to IP with anti-E-cadherin antibody, followed by immunoblotting with streptavidin-HRP (SA-HRP; upper panel) and anti-E-cadherin antibody (second panel). Control (Vo) and heparanase cells were subjected to cell fractionation as described in "Materials and Methods" and membrane fractions were subjected to immunoblotting applying anti-E-cadherin antibody (lower panel). Note reduced E-cadherin on the cell membrane of heparanase overexpressing cells. **(C)** Heparanase was added exogenously to FaDu cells for 4 h and the cells were then subjected to immunofluorescent staining applying anti-ɤ-catenin (left) and anti-β-catenin (middle) antibodies. JSQ3 nasal vestibule carcinoma cells were transfected with an empty vector (Vo) or heparanase gene construct (Hepa) and were subjected to immunofluorescent staining applying anti-β-catenin antibody. Scale bars represent 10 (left panels) and 30 (right panels) microns.

Supplementary Video 1 | T47D breast carcinoma cells (2×10^4) were plated in a 6-well plate in complete growth medium for 24 h. Cells were then serum starved for 6 h, six fields in each well were randomly selected and examined every 10 min for 18 h by a time-lapse system. Representative time-lapse movie is shown.

Supplementary Video 2 | T47D breast carcinoma cells (2×10^4) were plated in a 6-well plate in complete growth medium for 24 h. Cells were then serum starved for 6 h. Latent heparanase (1 μg/ml) was then added, six fields in each well were randomly selected and examined every 10 min for 18 h by a time-lapse system. Representative time-lapse movie is shown.

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