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Review

Heparan sulfate proteoglycans and heparin regulate melanoma cell functions☆

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ABSTRACT

Background: The solid melanoma tumor consists of transformed melanoma cells, and the associated stromal cells including fibroblasts, endothelial cells, immune cells, as well as, soluble macro- and micro-molecules of the extracellular matrix (ECM) forming the complex network of the tumor microenvironment. Heparan sulfate proteoglycans (HSPGs) are an important component of the melanoma tumor ECM. Importantly, there appears to be both a quantitative and a qualitative shift in the content of HSPGs, in parallel to the nevi–radial growth phase–vertical growth phase melanoma progression. Moreover, these changes in HSPG expression are correlated to modulations of key melanoma cell functions.
Scope of review: This review will critically discuss the roles of HSPGs/heparin in melanoma development and progression.
Major conclusions: We have correlated HSPGs' expression and distribution with melanoma cell signaling and functions as well as angiogenesis.
General significance: The current knowledge of HSPGs/heparin biology in melanoma provides a foundation we can utilize in the ongoing search for new approaches in designing anti-tumor therapy. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Melanoma is a malignant tumor that arises from melanocytes, dendritic cells embryologically derived from the neural crest. Melanocytes normally produce melanin, a pigment that defines the color of the skin and minimizes tissue damage from ultraviolet (UV) radiation [1]. In healthy skin, melanocytes reside mostly just above the basement membrane and in the hair follicles. The malignant transformation of melanocytes is a multistep process, which firstly results in the formation of benign nevi due to enhanced proliferation of melanocytes. Subsequently, some of the melanocytes aggregated to nevi exhibit dysplasia. The malignant transformation of melanocytes is completed during the radial growth phase (RGP), with the transformed cells showing intraepidermal proliferation. During the subsequent vertical growth phase (VGP), the melanoma cells acquire the ability to invade the dermis and subcutaneous tissue. Finally, the transformed cells may enter systemic circulation and reach distant organs through blood and lymphatic vessels [2]. Indeed, this tumor is characterized by a highly metastatic behavior and due to its neuroectodermal origin; patients with disseminated malignant melanoma frequently develop metastatic lesions in the central nervous system that can lead to fatal complications [3,4]. Epidemiological studies have characterized a family history of melanoma, multiple benign or atypical nevi, and a previous melanoma presentation as strong risk factors, subject to genetic predisposition. Moreover, immunosuppression, sun sensitivity and exposure to ultraviolet radiation are additional risk factors [5]. Alarmingly, the incidence of malignant melanoma is increasing at a very high rate compared to other human cancers [6]. According to the U.S. Cancer Statistics Working Group, skin cancer is the most common form of cancer in the United States. Indeed, melanoma, the third most common skin cancer following basal cell and squamous cell carcinomas, is the most lethal. In the United States in 2009, 61,061 individuals were diagnosed with melanomas of the skin of which 9154 succumbed to disease related complications [7]. Despite screening and early detection programs, the overall mortality rate from melanoma has remained stable or continues to rise. These data demonstrate how crucial it is to detect and record all the possible factors and their putative mechanisms of action, which facilitate the malignant transformation of melanocytes as well as the subsequent tumor growth and metastasis [8]. The optimal melanoma treatment depends on the stage of the disease. Surgical excision is the treatment of choice for early disease, while some patients at stages II and III may benefit from adjuvant therapy with interferon alfa (IFNa) [9,10]. Several novel

Abbreviations: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; GAG, glycosaminoglycan; ECM, extracellular matrix; PG, proteoglycan; MMPs, metalloproteinases; FN, fibronectin; CS, chondroitin sulfate; SDC, syndecan; GPC, glypican

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therapeutic approaches are under evaluation, some of which are already tested in clinical trials. Important examples are combined therapies with cytotoxic chemotherapy agents including dacarbazine (DTIC) and fotemustine with biochemotherapeutic agents like interleukin-2 [11]. Furthermore, the monoclonal antibody, ipilimumab, which specifically targets the cytotoxic T-lymphocyte antigen-4 blocking an immune-inhibitory pathway, was shown in a randomized clinical trail to be an effective cancer therapeutic [12]. Cytotoxic chemotherapy agents, such as dacarbazine, which is the standard approved treatment option for patients with advanced (stage IV) melanoma and fotemustine, have achieved disease regression in some cases [13,14]. However, actual cure, or containment of metastatic disease remains an unsolved problem. Drugs that target specific molecular abnormalities already identified in melanomas, seem the most advanced and promising weapons. Therefore, continuous study of mechanisms involved in the pathophysiology of melanoma, will hopefully point to an effective, more personalized strategy against this deadly threat.

2. The role of genetic abnormalities and classical pathway deregulation in melanoma development

Initiation and progression of melanoma have been associated with mutations in critical genes, which specifically control a range of important cellular processes [15]. Some of these mutations are gradually accumulated after the carcinogenic exposure to solar ultraviolet radiation — a mutation rate considerably higher than that of other solid tumors such as breast cancer or glioblastoma multiforme [16]. Specifically, the most common mutation, identified in melanoma cells is positioned in the protein kinase BRAF, which results in the substitution of valine with glutamic acid (V600E) of the protein product [2]. Ras mutations have been observed in approximately 15–20% of melanoma tumors [17], N-ras being the most frequently mutated member. Both of the above mutations cause constitutive activation of the serine–threonine kinases in the (extracellular signal-regulated kinase) ERK– (mitogen-activated protein kinase) MAPK pathway, which in turn participate in the regulation of cell growth, survival and angiogenesis, through a series of signaling cascades [18]. Apart from the MAPK pathway, N-Ras also stimulates phosphatidylinisitol-3-kinase (PI3K) to activate AKT, a serine/threonine-specific protein kinase whose downstream signaling regulates apoptosis, cell proliferation and tumor cell chemoresistance [19–21]. Cyclin D1 and cyclin-dependent kinases cdk4/cdk6 and cdk2 which are key mediators of cell cycle progression are frequently upregulated in melanoma [2]. Recently, the switch from RGP to VGP and the metastatic phenotype was associated with the loss of the AP-2α transcription factor. AP-2α regulates the expression of c-KIT, MMP-2, VEGF, and the adhesion molecule MCAM/MUC18, as well as two G-protein coupled receptors (GPCRs) PAR-1 and PAFR. The ligands for these GPCRs, thrombin and PAF, are secreted by stromal cells, emphasizing the importance of the tumor microenvironment in melanoma metastasis [22]. The KIT gene is another important gene for melanoma progression. It encodes a tyrosine kinase membrane receptor that stimulates the MAPK and PI3K pathways and it has proven to be very important for the migration of melanoma cells [23].

3. The remarkable cross-talk between melanoma cells and the surrounding ECM

3.1. Tumor ECM

The ECMs, insoluble and complex matrices, are responsible for the structure of the tissue architecture, as they provides mechanical support, especially in cartilage, bone, and tendons. Secondly, the ECM organizes its molecular components in varying distribution and geometry in order to produce or receive various signals, store and regulate the activity of growth factors and other signaling molecules, thus ultimately controlling cellular functions. ECM contains structural proteins including different types of collagens and elastin as well as glycosaminoglycans (GAGs); proteoglycans (PGs), molecules consisting of a protein core that is covalently modified with GAG chains; glycoproteins like laminin and fibronectin (FN); proteolytic enzymes that are able to cleave peptide bonds, such as the matrix metalloproteinases (MMPs); matricellular proteins, including thrombospondins and tenascins and other signaling molecules [24,25]. An important route of cell–ECM interplay is *via* matrix adhesion molecules and receptors such as integrins and discoidin-domain receptors [26] and another is through sequestration and activation of growth factors [27]. Therefore, one can conclude that during the course of a malignant disease, the ECM forms a frame around a growing tumor, either allowing, or impeding its spreading as the tumor cells attach, migrate through and finally exit its boundaries.

3.2. Heparan sulfate-containing PGs (HSPGs) — key component of tumor ECMs

Heparan sulfate-containing PGs (HSPGs) whose protein cores are modified with heparan sulfate (HS) chains are an important component of the ECM. HS chains are linear, negatively charged GAGs, which contain variably, sulfated repeating disaccharide units. The expression and modification of HS chains are related to their corresponding protein core [28]. HS biosynthesis is a multistep process beginning with a precursor macromolecule and evolving after diverse enzymatic transformation into a gross number of structurally heterogeneous products [29]. The consensus sequence for GAG addition to the protein core is the amino acid serine (the site of GAG attachment) followed by a glycine. The structures of the linkage tetrasaccharides connecting HS and CS chains to the core protein are identical. During biosynthesis, the selection of these sites for HS chain assembly depends on the activity of a-N-acetylglucosaminyltransferase I, which adds an N-acetylglucosamine (GlcNAc) moiety to the linkage tetrasaccharide substrate and commits it to HS synthesis [29–31]. HS chain elongation then proceeds through the sequential addition of specific disaccharide units. The disaccharide units contain two alternate sequences: N-glucosamine and uronic acid, either as β-D-glucuronic acid (GlcA), or epimerized by glucuronyl C-5 epimerase to α-L-iduronic acid (IdoA). The ester (O)-sulfation of C-6 glucosamine by 6-O-sulfotransferase and C-2 by 2-O-sulfotransferase of glucuronic acid or iduronic acid is very common, as well as the O-sulfation of C-3 of N-sulfated glucosamine. Glucosamine can be N-acetylated, or N-sulfated, which provides more sites for chain modification [30,31]. Moreover, in HS chains of different cellular origins, O-sulfation sites have been detected in the vicinity of the N-sulfation ones with varying incidence [32]. Importantly, HS chains, in a manner dependent on their sulfation pattern and size are able to increase HS specific-ligand binding [33]. Thus, in order for the growth factor–HS complex to demonstrate mitogenic activity the HS chains have to bear specific structural features [31].

HSPGs are located both at the cell membrane and within the ECM, particularly at the basement membrane. These PGs are able to interact with a variety of extracellular ligands including growth factors and adhesion molecules both through their HS and protein components. Through these interactions, HSPGs participate in key events including adhesion, migration, proliferation and differentiation, regulating cell fate. The most prominent basement membrane HSPGs are perlecan, agrin and collagen XVIII (mostly located at vascular and epithelial basement membranes). As key components of basement membranes in organs and tissues, they also participate in selective filtration of biological fluids, in establishing cellular barriers, and in modulation of angiogenesis. The ability to perform these functions is provided both by the features of the protein cores as well as by the unique properties of HS [34]. Thus, basal membrane, HSPG perlecan is especially involved in tissue development, differentiation and vasculogenesis, mediating cell/matrix interactions and growth factor storage and signaling [35,36]. With respect to cell membrane HSPGs, the two major cell membrane HSPG families are the syndecans (SDCs) and glypicans (GPCs), both

comprising a protein core substituted with 3–5 HS chains, but also chondroitin sulfate (CS) in some cases. It is mostly through the HS chains that HSPGs engage extracellular ligands, and the length and flexibility of HS allow the capture of distant or dilute ligands, making HSPGs ideal early sensors of changes in the extracellular environment. Indeed, they are able to act as low-affinity co-receptors to extracellular heparin binding proteins or endocytosis receptors [37–39]. Importantly, GPCs are anchored to the membrane by a GPI linkage, and therefore are limited to organizing ligands outside the cell [40]. By contrast, SDCs, a family of four HSPGs, consist of an ectodomain, a transmembrane domain, and a short cytoplasmic domain (with C1-proximal to the membrane, C2-distal to the membrane and V subdomains). This unique structure allows them to form constitutive homodimers, which means that they can organize formation of multimolecular complexes on the cytoplasmic face of the plasma membrane, in response to extracellular ligand binding [41,42]. Intracellularly, the highly conserved C1 and C2 domains mediate binding to cytoskeletal proteins especially the ERM proteins (ezrin/Radixin/Moesin). Moreover, the cytoplasmic domain of SDCs associates with syntenin, a protein that contains two PDZ domains, which enables the coupling of SDCs with cytoskeletal proteins, a process that leads to cell surface extensions [43]. The variable (V) region between C1 and C2 is unique to each syndecan [44]. Indeed, the V domain of SDC1 regulates cell spreading, as well as actin and fascin bundling [45]. The V domain of SDC2 plays a significant role in the organization of fibroblast matrix [46], whereas SDC4 binds phosphatidylinositol 4, 5 bisphosphate (PIP2) through its V domain, mediating SDC4 oligomerization and the activation protein kinase Cα (PKCα) [47].

Each syndecan member has been allocated discrete roles in the process of tumorigenesis. Thus, SDC1 collaborates with and regulates αvβ3 and αvβ5 integrin signaling, augmenting focal adhesion formation and regulating adhesion, invasion, metastasis and angiogenesis of tumor cells that express these integrins [48,49]. SDC2 has been found to bind to ezrin with the assistance of RhoA GTPase and indirectly organize the cytoskeleton by forming actin microspikes in fibroblast-like COS-1 cells [50]. Through these cytoskeletal changes it participates in the regulation of tumor cell motility [51,52]. Likewise, SDC4 has been found to regulate focal adhesion formations and thus regulate tumor cell motility [53] and spreading [54]. Indeed, the importance of HSPGs in cell–ECM communications is well illustrated by studies showing that various cell lines deficient in HS synthesis are unable to form stress fibers or focal contacts [55,56]. In summary, HSPGs conduct signals bidirectionally in and out of the cell, sequester proteins within secretory vesicles and link ECM proteins together, modulating thus, tissue homeostasis and processes regulating malignant transformation and progression [57]. It is worthwhile to note that HSPG participation is obligatory in classical pathway deregulation determined during melanoma development.

3.3. ECM modulations specific for the melanoma

The solid melanoma tumor consists of transformed melanoma cells, and the associated stromal cells including fibroblasts, endothelial cells, immune cells, as well as, soluble macro- and micro-molecules forming the complex network of the tumor microenvironment. There is increasing evidence that emphasizes the bidirectional interplay of the malignant cells with their supporting stromal cells [2]. Initially, associated cells can contain melanoma progression as it has been shown that keratinocytes have the power to partially restrain the malignant melanocyte transformation *via* the modulation of paracrine growth factors and cell adhesion molecules, such as E-cadherin, P-cadherin, desmoglein and connexins activity, which may result in controlled cell proliferation [58,59]. Modulations in the expression of these molecules, including cadherins, consecutively, enable the transformed cells to escape restraint by stromal cells [5]. Normally, the dermal ECM is rich in collagen I, mainly a product of fibroblasts. There appears to be a shift in the quality of the collagen matrix during disease progression as studies comparing primary melanomas to nevi, demonstrate that melanoma ECM exhibits fewer, but thicker collagen bundles, especially at the peripheral edges of the tumor [60]. In cutaneous melanoma and in accordance with phenotypic classification, there is either a great number of fibroblasts and fibrocytes and accumulation of fibrillar ECM components, as a result of epithelial–mesenchymal transition (desmoplastic phenotype), or atypical spindle cells with major proteoglycan accumulation (myxoid phenotype) [61]. Immunohistochemistry has also shown type IV collagen or laminin around tumor cells in the fibroplasia pattern of melanoma, which is seen mostly during the vertical growth phase of melanoma [62]. Overexpression of αvβ3 integrin has been shown to increase the expression of the antiapoptotic factor bcl-2 and the MMP-2, an endopeptidase, which facilitates invasion through enhanced degradation of the basement membrane. By activating this mechanism, melanoma progresses from RGP to VGP, and tumor cells are enabled to migrate through the vasculature [63–65]. Finally in invasive melanoma, there is an increased expression of highly specific ECM components including FN, SPARC, a basement membrane glycoprotein of the bone, tenascin C and laminins XY [66–69]. Importantly, an altered HS content has been established during melanoma progression pointing to a key role of HS in melanoma pathogenesis. Indeed, the switch from a low-risk radial to a high-risk vertical growth primary melanoma is characterized by significant changes in the expression of molecules involved in cell–cell: cell–ECM interface, as well as ECM integrity [70].

3.4. HSPG component of the melanoma ECM

In the melanoma model, HS demonstrates reduced sulfation and increased accumulation in B16 murine melanoma cells whereas, upregulated expression of cell-surface HSPGs in B16B15b murine melanoma cells has been connected with their high brain-metastatic behavior [71]. Both the synthesis and modifications of the HS chains bound into HSPGs are the cooperative result of many different enzymes, including sulfatases, glycosyltransferases, sulfotransferases and heparanases. These enzymes widen the complex spectrum of the HS chain properties modulating in turn cellular behavior. The aberrant modulation of several key HS biosynthetic enzymes resulting in specific changes of HS fine structures is directly correlated to efficient perpetration of invasion, proliferation, and metastasis by tumor cells [72]. The endosulfatases (Sulf-1 and -2), which remove the sulfate group at the 6-O sulfation sites are overexpressed in several cancers [73,74]. Indeed, the overexpression of Sulf-2 in high grade uveal melanoma has been correlated with poor disease prognosis [75]. Despite similar substrate specificity, Sulf-1 has mainly tumor suppressor functions whereas Sulf-2 presents tumor promoting functions [75] which is likely associated with the resulting fine alternations in HS binding affinities. Another crucial example of HS modifying enzymes is heparanase. Heparanase, the sole HS degrading endoglycosidase, is causally involved in inflammation, tissue remodeling, angiogenesis, and metastasis [76]. Correlation between heparanase expression and melanoma has been established. Thus, early studies report that melanoma heparanase is an enzyme with specificity for beta-D-glucuronosyl-N-acetylglucosaminyl linkages in HS, while elevated levels of heparanase have been detected in sera from metastatic tumor-bearing animals and malignant melanoma patients [77]. Indeed, heparanase expression is suggested to be correlated with the oncogenesis of melanoma as an increase in this enzyme expression has been reported along the benign lesion-cancer *in situ*-metastasis axis [78]. Moreover, heparanase is frequently expressed in oral mucosal melanoma, and its expression levels inversely correlate with the survival rates of this patient group [79]. Elevated levels of heparanase have also been associated with brain metastatic melanoma [80]. Through its endoglycosidase activity heparanase releases HS fragments of different molecular weights [81,82], as well as several ECM-resident growth factors with angiogenic and/or mitogenic properties [83,84]. Thus, bioactive HS cleaved from the melanoma cell-surface stimulated *in vitro* B16B15b cell migration but not proliferation, and importantly, enhanced *in vivo* angiogenesis [85]. The enhanced angiogenesis by these fragments was VEGF-

Table 1
The effect of HS on melanoma cell functions and the proposed mechanisms involved.

Cell line	Reported function	Suggested causative mechanism	Reference
B16B15b	↑ Migration & *in vivo* angiogenesis	Release of bioactive HS by heparanase cleavage	[85]
A375-SM	↑Focal adhesion formation & ↑ migration	Complex formation of SDC4 with α5β1 integrin induces PKCα activation	[91]
A375	↑ Migration	MC1R activation/inactivation of p38 induces an ↑ SDC2 expression (also in B16 & B16F10 cell lines)	[121]
SKMEL-178	↑Focal adhesion formation	HS & CS binding to YRLTVGLTRR, FN active sequence	[99]
M5	↑ Migration & ↓ adhesion	FGF-2-mediated SDC4 balanced expression/FAK activation	
SB1B BMM SBCL3 BMM	↑ Migration	Complex of SDC4/GEF-H1 & modulation by heparanase	[116]
Uveal melanoma	↑ HGF-mediated migration	SDCBP mediated activation of FAK/AKT/Src	[117]
MeCoP, MePA, MeOV, MeMO, MeTA	↑ Tumor growth & vascular maturation	Co-distribution: SDC1/VEGFR2	[146]
SK-MEL-24 SK-MEL-2	↑ Migration	EPAC/PI3K-mediated SDC2 translocation & HS production	[120]
70W	↑ Invasion	NT-mediated ↑ perlecan expression	[136]
	↑ Angiogenesis	Through a ternary complex with FGF-2 & FGFR	[71]
G361, UACC903, M93047, UACC647	↑ Invasion	SDC1, 4 mediated Wnt5A signaling	[136]
B16F10	↑ Network formation on Matrigel	SDC1 binding to AG73 laminin peptide	[102]
A2058 WM793	↑ Proliferation & migration	ERK/MEK-mediated heparanase & VEGF expression	[84]

independent but possibly due to signaling by some other heparin-binding growth factor(s). In addition, Roy and Marchetti [85] suggest that the increased vascularity seen with the HS bioactive fragments was due to their effects on the tumor microenvironment. The release of ECM-resident growth factors with angiogenic properties, such as the vascular endothelial growth factor (VEGF), facilitates their action. VEGF is a growth factor mostly known for its tumor angiopoietic activities; however, it also strongly induces tumor cell proliferation, invasion and metastasis through the up-regulation of specific transcriptional factors [84]. Previously, melanoma cells were found to overexpress VEGF receptors (VEGFR1 and 2) [86]. Interestingly, the expression of VEGF and heparanase is mutually up-regulated in melanoma, leading to increased proliferation and motility of melanoma cells. This co-expression is mediated through the activation of the MEK/ERK pathway [84]. The role of heparanase in the process of melanoma metastasis has been confirmed in early studies where treatment with heparanase inhibitors, such as N-acetylated N-desulfated heparin resulted in remarkable decrease of lung metastases in the mouse model [87]. As regarding heparanase action one must take into account concentration dependent effects, this being well illustrated on FGF-2 signaling. Extensive heparanase degradation of cell membrane HS chains on human metastatic melanoma cells (70W) inhibited FGF-2 binding [88]. These data suggest that there is a threshold, above which heparanase is able to suppress tumor growth, possibly since the extended elimination of HS can change the binding, and consequently the effect of several growth factors with heparin-binding sites [88]. Unexpectedly, treatment of 70W cells with low heparanase concentrations enhanced FGF-2 binding suggesting that low heparanase activity modulates HS structurally to increase affinity to FGF-2 [88].

4. The effect of HSPGs on melanoma cell functions

4.1. HSPGs affect the adhesion ability of melanoma

Melanocytes depend on integrins, heterodimeric cell surface receptors, to control their adhesive interactions with the extracellular matrix scaffold secreted by fibroblasts and keratinocytes. The integrin receptors allow cells also to sense the mechanical condition of the extracellular environment, responding by intracellular signaling, triggering cell survival, proliferation or migration events [89]. During melanoma development, changes in integrin expression, intracellular control of integrin functions and signals perceived from integrin ligand binding impact upon the ability of tumor cells to interact with their environment and enable melanoma cells to convert from a sessile, stationary to a migratory and invasive phenotype [90]. HSPGs have been assigned a key role on integrin functions [91]. Moreover, melanocyte transformation associated with substrate adhesion impediment demonstrated the role of glycosylation pattern in this process. Thus, no significant differences in cell surface expression of integrins were detected during the transformation process, but a clear electrophoretic migration shift, compatible with an altered glycosylation pattern, was observed for β1 integrin chain. Moreover, alterations both in PG glycosylation pattern and core protein expression were detected [92].

Integrin inside-out signaling modulates the adhesive affinity of the melanoma cells to specific ECM components, including FN, collagens and laminin [90]. Some of these ECM components are products of stromal fibroblasts and they are associated with greater motility of melanoma cells [93]. Indeed, FN a high molecular weight glycoprotein component of the ECM [94] has been associated to melanoma pathogenesis. Specifically, melanoma invasion, in a manner dependent on ERK/MAP kinase signaling has been associated with FN expression [95]. Importantly, FN carries sites for the binding of sulfated GAGs, membrane PGs and cell surface integrin receptors. The HSPG family, SDCs participate in this binding. Thus, SDC4 [96,97] and SDC1 [48,54] together with integrins, participate in the formation of focal adhesion sites regulating adhesion, invasion, metastasis and angiogenesis of tumor cells. Importantly, binding of A375-SM melanoma cells to FN through distinctive integrin heterodimers involves differential requirement for cell surface PG engagement [91]. Thus, αvβ1-dependent binding onto FN requires a PG co-receptor (SDC4), and α4β1-dependent does not. In addition the collaboration among αvβ1 and SDC4 resulted in an eightfold increase in protein kinase C alpha (PKCα) activation [91]. Indeed, genomic analyses trying to define the gene signature of the metastatic melanoma disease consistently identified β3 integrin, SDC4 and WNT5a genes [98].

SKMEL-178 melanoma cells utilize both their HS and CS chains to bind to the active sequence YRLTVGLTRR of the HBP/III5 domain in FN which induces focal-adhesion-promoting activity [99]. Importantly, during the adhesion of M5 melanoma cells onto FN, their FAK Y397 activation was correlated to SDC4 expression levels. Indeed it is suggested that a balance in SDC4 expression acts as a switch among adhesion and migration [100].

There are also interesting data about the HS-dependent interaction of melanoma cells with another ECM component, laminin. When melanoma cells treated with laminin were inoculated into murine model, metastases developed, in opposition with control cells and cells treated with FN [101]. AG73 (RKRLQVQLSIRT), a peptide from the G domain of the α1 laminin chain, has diverse biological activities with different cell types. The B16F10 melanoma cells were found to specifically bind to this peptide in a manner dependent on HS chains and the binding in turn facilitated the B16F10 network formation on Matrigel [102].

Specifically the HS side chains of SDC1 are suggested to be the binding partner for AG73 [102]. The effect of HS on melanoma cell functions and the proposed mechanisms involved are concisely summarized in Table 1.

4.2. HSPGs affect melanoma migration, invasion and metastasis

During malignant transformation of melanoma cells extraordinary alterations in their migratory and invasive properties are perpetrated. Melanoma cells produce proteolytic enzymes which degrade collagen I and elastin and expose integrin $\alpha v\beta 3$ binding sites, facilitating in this manner, the attachment of cells with the ECM, cell survival and eventually, metastasis [103–105]. A strong involvement of the HSPG component in these processes is implicated. Specifically a central role for SDC4 is proposed [100]. SDC4 shows a widespread expression both in growing and mature tissues, regulating cell motility through downstream intracellular signaling and actin cytoskeletal re-organization [106,97,107]. Thus, in the presence of phosphatidylinositol 4,5-bisphosphate (PIP2) and SDC4 core protein multimerization the activation of PKCα is perpetrated leading to alteration of actin cytoskeleton and subsequent formation of stress fibers and focal adhesions [108–110]. It is well established that SDC4 in collaboration with integrin $\alpha v\beta 1$, and through activation of FAK and Rho GTPase, create an adhesive phenotype. The enhanced adhesive status is reversed through the actions of tenascin-C, leading to increased motility with the extension of filopodia in fibroblasts, and increased tumor cell proliferation in glioblastoma and breast carcinoma [111,112]. Importantly, SDC4 is suggested to be a key effector of FGF-2-mediated melanoma cell migration and adhesion [100]. FGF-2 is an essential, constitutively expressed, autocrine factor in melanoma cells, which induces early radial growth phase and stimulates melanoma tumorigenesis [113–115]. Importantly, FGF-2 was shown to downregulate FAK Y397-phosphorylation during FN-mediated M5 cell adhesion, promoting their migration. The observed decrease in FAK Y397 activation was correlated to SDC4 expression levels. Thus, a balance in SDC4 expression perpetrated by FGF-2 may be required for optimal M5 cell migration [100]. The obligatory participation of discrete SDC4/1 levels in melanoma progression is confirmed in studies of brain metastatic melanoma (BMM) cell functions. In this study, the small GTPase guanine nucleotide exchange factor-H1 (GEF-H1) was identified as a new component of SDC signaling complex that is differentially expressed in BMM cells compared to corresponding non-metastatic counterparts. Thus, knockdown of GEF-H1, SDC1, or SDC4 decreased BMM cell invasiveness and GEF-H1 modulated small GTPase activity of Rac1 and RhoA in conjunction with heparanase treatment. Importantly, treatment of BMM with latent heparanase modulated SDC1/4 gene expression in a manner correlated to BMM cell invasion [116]. Moreover, the inhibition of syndecan-binding protein (SDCBP) expression by siRNA impaired the ability of uveal melanoma cells to migrate in a wound-healing assay. Indeed, silencing of SDCBP in mda-9/syntenin-high uveal melanoma cells inhibited the hepatocyte growth factor (HGF)-triggered invasion of Matrigel membranes and inhibited the activation of FAK, AKT and Src [117]. In addition to syndecan binding and recycling, SDCBP, a scaffolding-PDZ domain-containing protein, has been correlated to clustering of membrane receptors, intracellular trafficking, Sox4 activation, and signal transduction (reviewed in [118]). Mda-9/syntenin is able to influence the cell shape and also the migration and invasion ability of different types of cancer cells, including cutaneous melanoma [25–29] where high SDCBP expression has been related to metastatic spreading [25].

Increasing expression levels of another HSPG, SDC2, are documented in nevi and in human melanoma *versus* no expression in normal melanocytes of the human skin [119]. Importantly, SDC2 has also been shown to regulate the migratory/invasive properties of melanoma cells [119]. Thus, Epac, an effector molecule of cAMP, was suggested to increase the migration-dependent ability of melanoma cells through a heparan-sulfate related mechanism involving SDC2 [120]. SDC2 is also implicated in melanoma metastases in mice, where the overexpression of Epac, increases lung colonization of melanoma cells through SDC2 translocation to lipid rafts. Specifically, this translocation is facilitated by tubulin polymerization, through the Epac/phosphoinositol-3 kinase pathway [120]. The importance of SDC2 is further illustrated by a study showing that the overexpression of SDC2 enhanced migration and invasion of melanoma cells, whereas the opposite was observed when SDC2 levels were knocked down using small inhibitory RNAs [119]. Interestingly, the melanocyte-stimulating hormone (MSH), inhibition of melanoma cell migration and invasion is abolished through SDC2 overexpression [119]. Moreover, the melanocortin 1 receptor (MC1R) through inactivation of p38 MAPK was shown to regulate melanoma cell migration *via* enhanced SDC2 expression [121].

Limited data implicates, the second major cell membrane HSPG family, GPCs, in melanoma invasion. Thus, athymic mice that lacked GPC1 exhibited fewer pulmonary metastases following intravenous injection of murine B16-F10 melanoma cells [122]. Moreover, analysis of human malignant melanoma FNA samples has shown both high and zero expression GPC3 expression, which could be attributed to the type of antibody used against GPC3 [123,124]. Moreover, Nakatsura et al. (2008) and Nishimura et al. (2005) [125,126] suggest its targeting in GPC3 expressing melanomas, in view of the fact that GPC3 has shown anti-tumor immunogenic activity, as has already been done in phase I clinical trials for hepatocellular carcinoma.

The cell membrane CD44, whose extracellular domain may contain either HS or CS chains, additionally participates in the processes of melanoma migration/invasion. CD44 is a complex cell surface molecule, which takes part in cell migration, proliferation, differentiation, hematopoiesis and apoptosis. Due to genetic and post-translational modifications, the structure and the final role in tumorigenesis of this molecule vary according to tumor cell type [127,128]. CD44 is the main hyaluronan binding receptor but it also collaborates with MMPs and pro-angiogenic factors, such as VEGF and FGF-2. Moreover, its cytoplasmic domain induces specific intracellular signaling pathways through interactions with the actin cytoskeleton [129–131]. CD44 is suggested to be an unreliable marker of melanoma prognosis, as its contribution in tumor progression was judged to be controversial [132]. Thus, its decreased expression has been correlated with poorer prognosis in stage I human cutaneous melanoma. This association could be partly attributed to the fact that reduced levels of CD44 allow spreading of the localized tumor [133]. However, a separate report suggests that the enhanced expression of CD44 in melanoma metastatic tumors enhanced a pro-migratory phenotype [134]. Its positive effect on melanoma cell motility is supported by a study on SCID mice injected with the human melanoma cell line melanoma SMMU-2, where treatment with an antibody against CD44 suppressed melanoma metastasis [135].

Basal membrane HSPGs are also implicated in the processes of melanoma invasion [136]. Indeed, during the process of malignant melanocyte transformation associated with substrate adhesion impediment, perlecan protein core expression levels were found to be upregulated [92]. Perlecan was found to be particularly deposited to the pericellular matrix of melanoma cells and its expression levels were correlated with greater invasiveness. Interestingly, perlecan mRNA levels were upregulated within 10 min of neurotrophin stimulation, indicating that perlecan is an early response gene. This upregulation also occurred prior to heparanase production, suggesting that perlecan expression and its regulation might play a pivotal role in the initial onset of invasion [136]. Moreover, perlecan as well as SDC1 were identified as targets of heparanase activity in melanoma cells with implications in the control of melanoma cell invasion and metastasis [71]. Reduction of perlecan expression downregulated the malignant phenotype in melanoma clones [36]. In tumor allografts induced by highly invasive mouse melanoma cells, perlecan suppression caused substantial inhibition of tumor growth and neovascularization [35]. The roles of HSPGs on melanoma cell functions are schematically presented in Fig. 1. Indeed, HSPGs modulate key intracellular signaling pathways, including PKCa, Erk or Akt

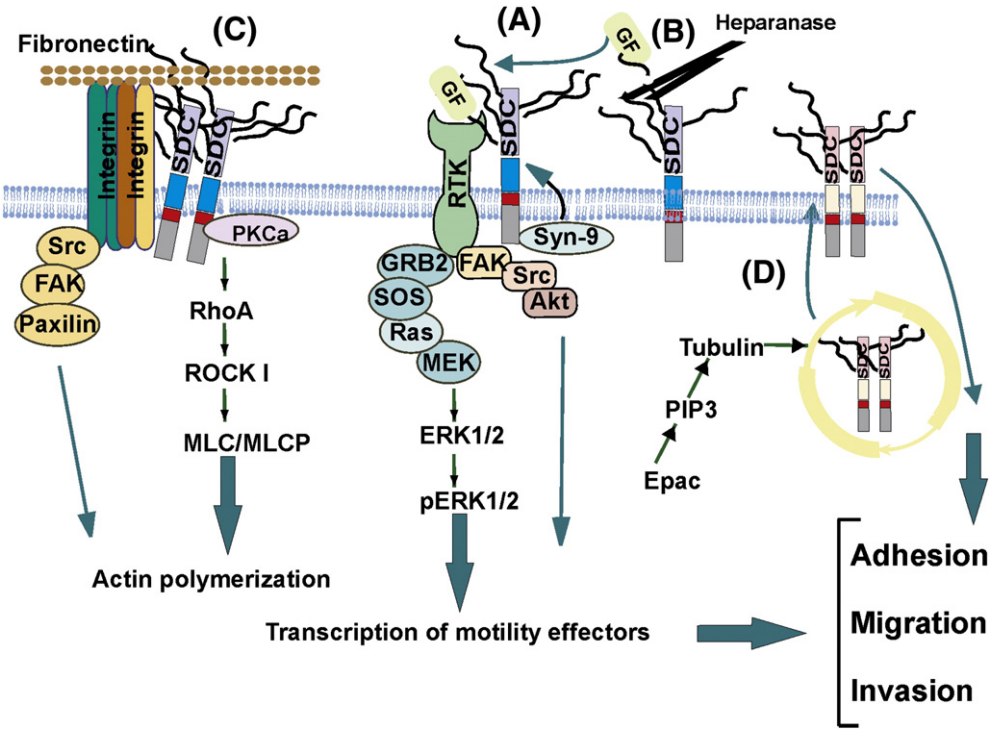

Fig. 1. Schematic presentation of the roles of HSPGs on melanoma cell functions. (A) HSPGs facilitate binding of growth factors (GFs) to their respective receptor tyrosine kinases (RTKs) and activation of downstream signaling; (B) heparanase cleaves HS-bound growth factors to regulate their downstream signaling; (C) upon binding to FN, SDC-4 molecules polymerize, form complexes with specific integrin members to activate intracellular signaling resulting in actin polymerization and focal complex formation; (D) through Epac/PIP3/tubulin dependent mechanism SDC-2 is translocated to lipid rafts to regulate melanoma cell migration.

signaling, which ultimately results in aggressive adhesion, migration and invasion properties of melanoma cells.

5. Angiotropic behavior of melanoma cells—role of HSPGs

Angiogenesis is the formation of a new vascular network among pre-existing vessels. It is a process that leads to tumor growth and metastasis, both by feeding the tumor with oxygen and necessary nutrients and also by providing tumor cells with escape routes from the primary site of growth [137]. Even though melanoma cells prefer lymphatic vessel spreading, angiogenesis alone supports disease. Indeed, angiogenesis is crucial during the vertical growth phase of the tumor. Importantly, melanoma cells have developed an additional extravascular way of migrating towards distant target organs defined as neurotropism. Neurotropism (the ability of melanoma cells to travel along nerves) is a phenomenon long known, that characterizes melanoma spreading. Likewise, the study of angiotropism (melanoma cells migrating along the external surface of vessels) has added interesting data, bringing scientists closer to an efficacious therapeutic strategy [138]. In more detail, melanoma cells form an angio-tumoral complex, adhering to the endothelium *via* a laminin matrix and this procedure is called "extravascular migratory metastasis" (EVMM) [138].

Heparanase affects angiogenesis in melanoma regulating the interaction of HS bioactive fragments and the autocrine/paracrine factors of melanoma [139,83]. Autocrine and paracrine growth factors secreted by the melanoma solid tumor such as VEGF, FGF-2, platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) can promote the creation of neovasculature [140] whereas, interleukin-8 (IL-8), which is stimulated by TGF-β, ultraviolet B radiation and hypoxia can regulate vascular permeability [141]. HS constitutes both low-affinity FGF-2 receptors and co-receptors in the complex of the high-affinity FGF-2 receptor FGFR. Additionally, they can affect FGF-2/FGFR dimerization by transphosphorylation of the receptor and signaling [142,143]. Heparanase degradation of cell surface HS in a concentration dependent manner can both augment and inhibit FGF-2-mediated angiogenesis [87]. These data suggest that the activation of FGF-2 downstream signaling pathways, including ERK and FAK phosphorylation [144,145], depends on whether cleaved HS is attached to cell surface HSPGs or exists in a soluble form. Interestingly, FGF-2 signaling through ERK or FAK is not activated in cells with heparanase deficiency [88].

It is worthwhile to note that SDC1 is accumulated within the extracellular matrix in melanoma where it is co-localized with vascular endothelial growth factor receptor 2 (VEGFR2). Indeed, its extracellular domain has been implicated in vascular maturation and growth of melanoma [146]. One mechanism for its pro-angiogenic activity is possibly through binding of its ectodomains with αvβ3 and αvβ5 integrins, as shown in human mammary carcinoma [48]. In metastatic melanomas, perlecan levels are found to be upregulated in a manner associated with a more aggressive phenotype [136]. Perlecan is able to bind to FGF-2 and acts as a low-affinity/high-capacity receptor, playing this way an important role in angiogenesis [147]. Subsequently, the down-regulation of perlecan expression seems to inhibit the growth and angiogenesis in melanoma [35]. Indeed, the degradation of perlecan HS chains by heparanase results in the release of bioactive growth factors which later attach to cell receptors and stimulate angiogenesis, growth, migration, and invasion [71].

6. The effects of heparin on melanoma progression

Heparin, a highly sulfated and charged GAG, is released by connective-tissue-type mast cells upon activation and has been implicated both in inflammatory and defense mechanisms [148,149]. This GAG is subject to multiple conformational changes largely attributed to the domination of L-iduronic acid residues, which bestow to heparin the ability to engage in complex protein interactions. Indeed, heparin exerts most of its activities through highly specific electrostatic interactions with heparin-binding factors, including growth factors and chemokines, a quality which is largely attributed to the domination of

Table 2
Heparin actions in various melanoma cell lines.

Cell line	Reported function	Suggested causative mechanism	Reference
WM9	↑ Proliferation	Participation in FGF-2 signaling pathway (also in M5 cell line)	[164]
M5	↓ Adhesion	Heparin internalization/increased p53 expression/downregulation & deactivation of FAK	[171]
B16F10	↓ Proliferation & angiogenesis	Reduced expression of angiogenic factors	[119]
MV3	↓ Migration	Binding & reduction of CYR61 blocking of CYR61/VLA-4 signaling axis	[169]
A375	↓ Adhesion	2-O,3-O desulfated heparin mediated inhibition of P-selectin binding	[170]
		Actin cytoskeleton changes downregulation & nuclear redistribution of PKCα (also in M5)	[172]

L-iduronic acid residues [150,57,151,152]. Importantly, the pattern of sulfation in heparin is crucial for the determination of its final characteristics, both in terms of coagulation and cancer and especially in integrin-mediated adhesion of tumor cells [153].

The processing of heparin, in addition to unfractionated heparin, results in the formation of different active derivatives including low molecular weight heparin (LMWH), which is obtained through chemical or enzymatic depolymerization of heparin or fondaparinux, a synthetic heparin pentasaccharide based on the heparin/AT binding site [154–156].

Heparin is principally known for its anticoagulant effect. It forms a complex with antithrombin III (AT) through a pentasaccharide sequence, leading to indirect inhibition of thrombin, factor Xa, as well as factors IIa, XIIa, XIa, and IXa [155]. Since 1960, heparin has been widely used in clinical practice, for the prevention and treatment of thromboembolism [157,158].

The antitumor activities of heparin through both its anticoagulant and non-anticoagulant features have been widely established. Thus, LMWH is broadly used to prevent venous thromboembolism, a fatal complication of malignancy, ultimately improving patient survival rate [159]. On the other hand its non-anticoagulant abilities are known to inhibit tumor growth, metastasis and angiogenesis [160]. Thus, endogenous mast cell-derived heparin has shown a great suppression of human breast cancer growth, when co-cultured with fibroblasts [161]. Moreover, heparin is capable to suppress metastasis in various types of cancer, like breast cancer and melanoma [162]. Restraining of angiogenesis is partly responsible for its anti-metastatic effects. This is achieved through VEGF, tissue factor (TF), and platelet activating factor inhibition, as well as through reduced autocrine expression of vascular permeability factor VPF/VEGF [163]. Heparin also decreases adhesion of malignant cells to vascular endothelium, mostly through binding and deactivation of P-selectin. In this manner, it inhibits the rolling stages of cell adhesion, which further impedes cell migration. Moreover, heparin downregulates the activity of molecules which are important for metastasis, including MMPs, serine proteases and heparanases [163]. Another important mechanism of heparin action is the inhibition of fibrin strand formation around circulating tumor cells, making them vulnerable to mechanical destruction and host immune cell attack [162].

6.1. The role of heparin on melanoma cell functions

The participation of heparin in melanoma cell proliferation signaling pathways seems cell specific and depends on the interaction with other heparin-binding proteins. Thus, heparin takes part in FGF-2-induced mitogenic effects in WM9 and M5 human melanoma cells. However, exogenous heparin showed a mild inhibition of melanoma cell proliferation [164]. Likewise, LMWH, as well as dp18, an oligosaccharide heparin fragment without anticoagulant features have demonstrated a negative effect on murine and human melanoma cell proliferation [165,166]. On the other hand, neither UFU nor LMWH exerted any effects on the growth of HT168-M1 human melanoma cells [167].

Heparin has principally shown anti-adhesive qualities in melanoma preclinical studies. The integrin VLA-4-mediated binding is important for the metastatic dissemination of melanoma cells. Recently heparin was found to possess a binding capacity to VLA-4. LMWHs efficiently blocked VLA-4 cell binding, dominantly *via* the integrin's α-chain. These data could contribute to the heparin function to attenuate metastasis in a selectin-dependent manner [168]. This signaling axis has been shown to involve cysteine-rich angiogenic inducer 61 (CYR61), a matricellular protein, which is produced by and implicated in the functions of various types of tumors. Investigation of the possible roles of LMWH tinzaparin in MV3 melanoma cell metastasis has shown that tinzaparin through binding to CYR61 and suppression of its free cellular secretion, blocks the signaling axis of CYR61/VLA-4 [169]. Likewise, heparin takes part in P-selectin-mediated melanoma cell adhesion. Specifically, 2-O,3-O-desulfated heparin was shown to inhibit the P-selectin-mediated A375 human melanoma cell adhesion [170]. Heparin has interestingly demonstrated suppressed adhesion and migration of melanoma cells on FN substrate through its internalization and subsequent activation of p53/FAK signaling axis, which results in downregulation and deactivation of FAK, a key regulator of melanoma motility [171]. Additionally, LMWH seems to alter actin cytoskeleton and block the adhesion of M5 and A375 melanoma cells on FN through downregulation and nuclear redistribution of PKCa in a dose dependent way [172]. These data demonstrate parallel pathways of action through which heparin and its derivatives attenuate tumor cell adhesion and can at least partly explain their anti-metastatic activities [169]. Mechanisms utilized by heparin and derivatives to regulate melanoma cell functions are summarized in Table 2.

The observed effects of heparin on melanoma cell functions result in anti-metastatic action. Thus, LMWH reduces the number of metastases when HT168-M1 melanoma cells are injected into SCID mice [167]. There are also studies using B16 and B16-BL6 melanoma murine models of metastasis, which confirm that the non-anticoagulant form of LMWH (NA-LMWH), tinzaparin, very low molecular weight heparin (VLMW-H) and a low molecular weight heparin with 100% succinylation of desulfated N groups (Succ100-LMW-H) inhibit melanoma metastases [173–175]. Likewise, after specific processing, oligosaccharide fragments of heparin with non-anticoagulant abilities have shown suppression of melanoma metastasis [166]. Heparin is suggested to affect FGF-

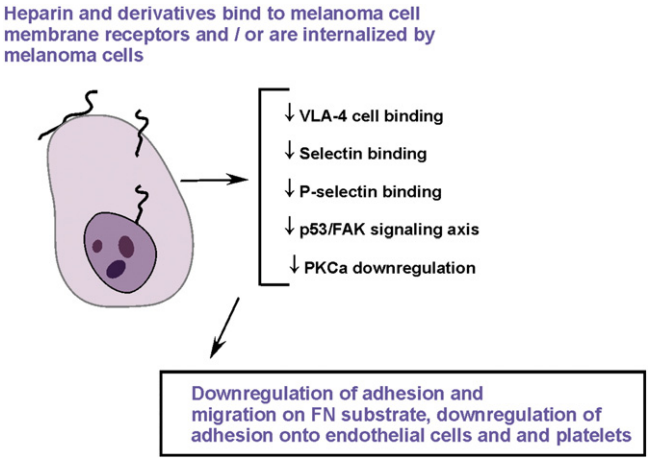

Fig. 2. Schematic presentation of the effects of heparin and its derivatives on melanoma cell functions as well as of mechanisms involved.

2-signaling pathways, strongly implicated in melanoma metastasis. In order to neutralize FGF-2 activity, the use of a heparin binding peptide for FGF-2 in a B16-BL6 murine melanoma model achieves inhibition of both neovascularization and metastasis, indicating a possible role for heparin in these specific FGF-2-dependent signaling pathways [176].

The role of heparin in melanoma angiogenesis seems to be controversial. Even though there are studies that demonstrate stimulation of angiogenic factor activity, its main role is considered to be inhibitory. While in earlier studies heparin seems to participate in the interaction of VEGF with its cell surface receptors in melanoma cell lines [177], recently, a chemical conjugate of low-molecular-weight heparin and deoxycholic acid has shown anti-angiogenetic properties in melanoma tumors, through downregulation of the expression of angiogenic factors like VEGF, heparanase and MMPS, thus inhibiting neovascularization [165]. On the other hand, the overexpression of TF, a factor known to mediate the anti-angiogenetic properties of heparin and the production of which is reduced by heparin through TFPI release, did not increase the expression of VEGF and also did not lead to angiogenesis in human melanoma cells [178]. The roles of heparin and derivatives on melanoma cell functions are schematically presented in Fig. 2. Thus, heparin and derivatives by effecting tumor cell selectin-dependent binding, their PKCa activation as well as p53/FAK signaling axis downregulate adhesion to platelets and endothelial cells which results in augmented metastasis.

7. Present strategy in utilizing HSPG/heparin properties for targeted therapy

HSPGs have repeatedly been used as potential therapeutic targets in murine metastatic melanoma models with remarkable results. In the majority of these studies, an increase in HSPG expression resulted in enhanced metastatic ability, whereas downregulation of their expression and/or inhibition of their activities have lead to significant reduction in melanoma metastases. Prominent examples exploiting this approach are the use of a CD44 antibody, antisense perlecan DNA or GPC1 knockouts [135,36,122]. Moreover, a vaccine against GPC3, an oncofetal antigen, is proposed to be tested in phase I clinical trials [123]. However, various amplifying and suppressing effects have been attributed to HSPGs as far as cancer development and progression are concerned. Thus, apart from the apparent inhibition of metastasis, their elimination could possibly induce a number of changes that would complicate the overall health status and long term outcome of the murine model used [179]. Interestingly, a novel HS mimetic, M402, targeting various tumor-host cell interactions has shown anti-metastatic and anti-angiogenic effects in an experimental metastasis model [180]. As heparanase is correlated with tumor growth, angiogenesis and metastasis, targeting this enzyme would be a promising therapeutic approach against melanoma. Transfection of melanoma cells with the anti-heparanase ribozyme or siRNA vectors has resulted in significant reduction of adhesion and invasion *in vitro* and vascularization and metastasis *in vivo* [181]. In search for fewer side effects, a lentiviral miR30-based RNA interference against heparanase has shown suppression of melanoma metastasis with lower liver and lung toxicity [182]. Another molecule that blocks heparanase and results in antitumor effects is heparin. In murine metastatic melanoma models, specifically sulfated and sized heparins have shown an interesting metastasis inhibition [173–175,166]. Indeed, heparin is implicated in a wide field of cell procedures, affecting systemic defense and coagulation cascades, yet its use comes with additional side effects. Further, specific processing of this multifunctional molecule could potentiate its antitumor effects, to obtain maximum results. The roles of heparin and derivatives on melanoma cell functions are depicted in Fig. 2. Shortly, heparin and derivatives specifically inhibit VLA-4 and selectin-dependent melanoma cell binding as well as the p53/FAK signaling axis.

8. Conclusions

HSPGs are suggested to be crucial effectors of the tumor microenvironment, able to determine melanoma development and progression. The effects of HSPGs are clearly dependent on the specific correlation among their expression, distribution and melanoma stage. Importantly, HSPGs seem to be permissive “conduits” at the tumor cell/ECM interface, mediators of signals supporting melanoma disease progression. The emerging mechanisms could potentially be exploited for designating discrete HSPGs as therapy target for specific melanoma grades. First steps have been made in this direction. Promising examples exploiting this approach are the use of a CD44 antibody, antisense perlecan DNA or GPC1 knockouts as well as a trail vaccine against GPC3. Potential candidates could also be SDC1, 2 and 4, as a pattern correlating their expression and malignant melanoma behavior has been identified. Design of new non-anticoagulant heparin derivatives would facilitate their anti-metastatic effects while augmenting adverse side effects. Along these lines, the available data could provide a basis in order to deepen our understanding of HSPGs/heparin in melanoma progression.

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