LUND UNIVERSITY

Biological Functions of Iduronic Acid in Chondroitin/Dermatan Sulfate in Tumor and Brain Development

Thelin, Martin

2012

Biological Functions of Iduronic Acid in Chondroitin/Dermatan Sulfate in Tumor and Brain Development

Martin Thelin

Doctoral dissertation

Bye due permission of the Faculty of Medicine, Lund University, Sweden to be publicly defended in Rune Grubb lecture hall, BMC, Lund on Friday 28th of September 2012, at 13.00

Faculty Opponent

Professor Liliana Schaefer

Institute of General Pharmacology and Toxicology, Goethe-University of Frankfurt/Main

LUND
UNIVERSITY

Organization	Document name
LUND UNIVERSITY Dept. of Experimental Medical Science BMC D12, 221 84 Lund	DOCTORAL DISSERTATION Date of issue 2012-09-28
Author(s) Martin Thelin	Sponsoring organization

Title and subtitle

Biological Functions of Iduronic Acid in Chondroitin/Dermatan Sulfate in Tumor and brain development

Abstract

Cell behavior such as migration and proliferation, especially during cancer development, are balanced by the surrounding environment. Complex polysaccharides called glycosaminoglycans (GAGs) are part of this environment and they are known to modulate tumor development. One of these GAGs, dermatan/chondroitin sulfate (CS/DS), is attached to proteins either bound to the cell surface or secreted in the extracellular space. CS/DS is a long linear polysaccharide consisting of alternating disaccharide units (i.e. N-acetylgalactosamine and glucuronic acid/iduronic acid). CS/DS is a very dynamic structure, as it can be greatly modified by epimerization and sulfation. Epimerization is catalyzed by two enzymes, DS-epimerase 1 (DS-epi1) and 2 (DS-epi2), resulting in the formation of iduronic acid (IdoA). The aim of this thesis was to investigate the role of iduronic acid in cancer and brain development.

The first part of this thesis gives a general review of the biosynthesis of CS/DS, tumorigenesis and finally intertwines these fields. It also comprises the role of CS/DS in neuritogenesis. The present investigation section presents the potential role of DS-epimerase 1 in tumor biology with emphasis on migration of tumor cells. Downregulation of DS-epi1 in a esophageal cancer cell line results in reduced invasion and migration. This effect was found to be partially mediated by presentation of hepatocyte growth factor to the MET receptor by IdoA. Furthermore, DS-epi1-silenced cells had malfunctioning disassembly of adhesion complexes and abnormal cytoskeleton architecture. During brain development, DS-epi2 is known to be highly expressed. We generated a DS-epi2-deficient mice, which displayed no anatomical, histological or morphological abnormalities in brain.

In summary, this thesis have identified new functions for IdoA in tumor development, which maybe of potential use in generation of novel therapeutics.

Key words: DS-epimerase 1, DS-epimerase 2, iduronic acid, chondroitin sulfate, dermatan sulfate, hepatocyte growth factor, MET receptor, cancer, esophagus squamous cell carcinoma

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title 1652-8220	ISBN 978-91-87189-37-1
Recipient´s notes / Number of pages: 140	Price
Security classification	

Biological Functions of Iduronic Acid in Chondroitin/Dermatan Sulfate in Tumor and Brain Development

Martin Thelin

LUND
UNIVERSITY

From:
Department of Experimental Medical Science

Faculty and Department
ISBN 978-91-87189-37-1
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series
2012:74

To Katrin

Table of contents

List of publications

Paper I
Thelin MA, Svensson KJ, Shi X, Bagher M, Axelsson J, Isinger-Ekstrand A, van Kuppevelt TH, Johansson J, Nilbert M, Zaia J, Belting M, Maccarana M, Malmström A. (2012)
Dermatan Sulfate Is Involved in the Tumorigenic Properties of Esophagus Squamous Cell Carcinoma. Cancer Res. 2012 Apr 15;72(8):1943-52.

Paper II
Bartolini B, **Thelin MA**, Rauch U, Feinstein R, Oldberg A, Malmström A, Maccarana M. (2012)
Mouse development is not obviously affected by the absence of dermatan sulfate epimerase 2 in spite of a modified brain dermatan sulfate composition. Glycobiology. 2012 Jul;22(7):1007-16.

Paper III
Bartolini B*, **Thelin MA***, van Kuppevelt TH, Malmström A, Maccarana M.
The role of dermatan sulfate in migration of aortic smooth muscle cells. Manuscript.

**equal contribution*

Paper not included in the thesis

Malmström A, Bartolini B, **Thelin MA**, Pacheco B, and Maccarana M
Iduronic acid in chondroitin/dermatan sulfate: biosynthesis and biological function. Journal of Histochemistry & Cytochemistry. 2012 Aug 16 [Epub head of print].

Abbreviations

GAG	glycosaminoglycan
ECM	extracellular matrix
CS/DS	chondroitin/dermatan sulfate
HA	hyaluronic acid
PGs	proteoglycans
ST	sulfotransferase
Xyl	xylose
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcA	D-glucuronic acid
IdoA	L-iduronic acid
VEGF	vascular endothelial growth factor
EGF	epidermal growth factor
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
TGF-beta	transforming growth factor-beta
MMP	matrix metalloproteinases
CAFs	cancer-associated fibroblasts
AoSMC	aortic smooth muscle cells
CNS	central nervous system
ESCC	esophageal squamous cell carcinoma
DS-epi1	dermatan sulfate epimerase 1
DS-epi2	dermatan sulfate epimerase 2
D4ST-1	dermatan 4-*O*-sulfotransferase 1
C4ST-1	chondroitin 4-*O*-sulfotransferase 1

Introduction

In order to develop novel therapeutics, we need to understand the biological processes. Cell behavior such as migration and proliferation are critical processes during cancer development and are balanced by the surrounding environment. Complex polysaccharides called glycosaminoglycans (GAGs) are part of this environment and they are known to modulate tumor development, for example by growth factor sequestering, by functioning as co-receptors as well as mediating cell adhesion during migration and invasion.

One of these GAGs; dermatan/chondroitin sulfate (CS/DS), is attached to proteins either bound to the cell surface or secreted in the extracellular space. CS/DS is a long linear polysaccharide consisting of alternating disaccharide units (i.e. N-acetylgalactosamine and glucuronic acid/iduronic acid). CS/DS is highly variable owing to its modifications, namely epimerization and sulfation. During biological processes such as tumor and brain development, both the amount and the degree of sulfation and epimerization of CS/DS is altered.

The aim of this thesis was to understand the role of one of these modifications, i.e. the epimerization, in cancer biology and brain development. Epimerization is catalyzed by two enzymes, DS-epimerase 1 and 2. The first part of this thesis gives a general review of the biosynthesis of chondroitin/dermatan sulfate, tumorigenesis and finally intertwines these fields. It also comprises the role of chondroitin/dermatan sulfate in neuritogenesis. The second part of the thesis, present investigation, presents the potential role of DS-epimerase 1 in cell migration and tumor biology. Furthermore, it reports of the involvement of DS-epimerase 2 in brain development. These findings bring the DS-epimerases into a new biological focus.

Background

Traditionally, the extracellular matrix (ECM) has been viewed as a matrix that supports and organizes a tissue. During the last decades, new functions have emerged, where the ECM has been shown to have a number of regulatory functions that influence cell behavior. For example, ECM influences proliferation, migration, and differentiation. These functions are mediated by the effects of the ECM on cell-surface receptors, such as presentation and sequestering of growth factors. Complex polysaccharides often participate in these events, mainly due to their growth factor binding abilities. Hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), midkine, pleiotrophin, and Wnt are all influenced by these intricate polysaccharides. A number of differently composed polysaccharides, glycosaminoglycans (GAGs), have been described, i.e. dermatan/chondroitin sulfate (CS/DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronic acid (HA). CS/DS, HS, and KS are covalently attached to core proteins, forming proteoglycans (PGs). HA, on the other hand, is a large molecule (10^2–10^4 kDa) devoid of core proteins. HS and CS/DS contain the same protein linkage region and they both exist on secreted and cell membrane-bound PGs. Interestingly, some PGs can carry both HS and CS/DS, such as syndecans and CD44.

Almost a century ago, a specialized form of heparan sulfate, heparin, was characterized. It is an anticoagulant drug, which underscores the biological importance of GAGs. A number of animal studies have shown that heparin inhibits cancer development, and clinical trials on lung cancer are ongoing. HS and HA are extensively studied GAGs with known key regulatory functions in a number of pathological conditions, including tumorigenesis and inflammation. HS-PGs such as syndecans, glypicans, and perlecan have been shown to modulate angiogenesis, proliferation, and metastasis, which are essential events in tumor progression. CS/DS, on the other hand, have been less investigated although the functions of CS/DS often overlap with those of HS. CS/DS-PGs are generally considered to be an extracellular growth factor binding reservoir whereas HS-PGs mediate the biological functions. However, novel regulatory functions of CS/DS and CS/DS-PGs are emerging.

Chondroitin/dermatan sulfate

CS/DS biosynthesis and structure

Chondroitin sulfate (CS) is a long polysaccharide consisting of repeating disaccharide units, i.e. D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) attached to a serine in core proteins. CS chains contain enormous amounts of biological information. They can be greatly modified by epimerization and sulfation (1). In total, eleven enzymes participate in the polymerization whereas ten enzymes are involved in CS chain modifications.

In dermatan sulfate (DS), GlcA is converted to L-iduronic acid (IdoA) by two DS-epimerases. Thus, after conversion of GlcA to IdoA, the polysaccharide is named DS. The content of IdoA varies greatly in the polysaccharide chain, from a couple of percent to being the predominant residue. The presence of IdoA has been overlooked in many cases, and the polysaccharide is therefore often named CS in the literature (Table I). In this thesis, no distinction between CS and DS will be made; instead, it will be termed CS/DS.

Polymerization

The formation of the CS/DS chain starts after formation of the linkage region, which is composed of GlcA-Gal-Gal-Xyl attached to a serine residue of the core protein. As the linkage region is identical in HS and CS/DS, the introduction of the first GalNAc determines that a CS/DS chain will be synthesized. Six CS/DS polymerases have been identified, and they are ubiquitously expressed in humans. The importance of CS/DS was demonstrated in *C.elegans,* as knockdown of CS/DS polymerases resulted in defect cell division (2).

Epimerization

DS-epimerase 1 (DS-epi1) and DS-epimerase 2 (DS-epi2) catalyze the formation of IdoA by repositioning the C5 carboxyl group in space. This generates the stereoisomeric isoforms of GlcA i.e. IdoA. DS-epi1 and DS-epi2 are both ubiquitously expressed and have structural features in common. They are encoded by two different genes. DS-epi1 and DS-epi2 are located on chromosomes 6 and 18, respectively. DS-epi1 contains two domains whereas DS-epi2 carries an additional, third domain (figure 1).

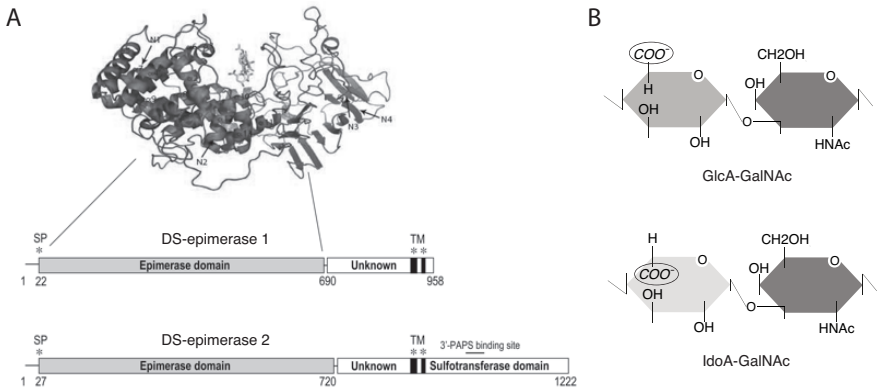


Figure 1. DS-epimerases and IdoA conversion. *A,* Structure domain of DS-epi1 and DS-epi2. *B,* structure of GlcA- and IdoA disaccharides.

The epimerase domain is located at the N-terminus while the functions of the C-terminal domain (DS-epi1) and central domain (DS-epi2) are unknown. Interestingly, the primary sequence of the C-terminal domain of DS-epi2 resembles that of O-sulfotransferases, including the binding site of the sulfate donor (i.e. 3'-phosphoadenosine 5'-phosphosulfate). By structural modeling and site-directed mutagenesis of DS-epi1, a possible catalytic mechanism has been put forward. This starts with removal of a proton by a histidine, followed by cleavage of the GlcA-GalNAc linkage. Thereafter, a histidine located on the opposite side of the sugar functions as a general acid and protonates the intermediate sugar, and the final step involves re-formation of the glycosidic linkage (3).

The epimerase reaction is reversible, with an equilibrium of 9:1 (GlcA versus IdoA) in *in vitro* conditions when the biosynthetic complex has been detergent-solubilized (4). Interestingly, *in vivo* CS/DS chains can contain a higher proportion of IdoA, which is explained by the strong relationship between 4-O-sulfation and IdoA (4). It has been proposed that 4-O-sulfation of the GalNAc flanking IdoA prevents back-epimerization. In support of this theory, transient downregulation of dermatan 4-O-sulfotransferase 1 (D4ST-1) results in reduced IdoA content (5).

In the CS/DS chain, IdoA can be differently distributed. IdoA can either be situated in blocks (stretches of ≥ 12 residues), in alternating IdoA/GlcA structures, or in isolated IdoA units flanked by GlcA residues (Figure 2).

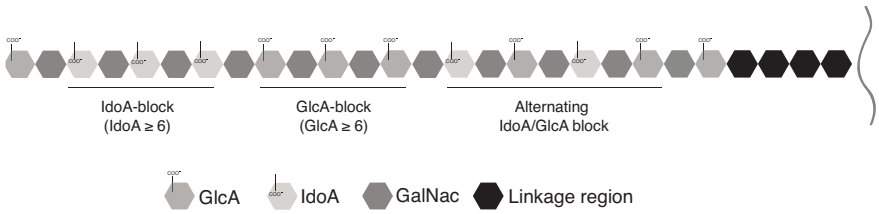


Figure 2. IdoA distribution in CS/DS. IdoA is present in clusters, IdoA-blocks, or as interspersed units i.e. alternating IdoA and GlcA units.

The biological function of IdoA has been studied in a loss-of-function model. DS-epi1-deficient mice were smaller than WT mice and in addition, DS-epi1-/- mice presented a changed skin architecture with larger skin collagen fibrils as compared with WT littermates (6). Interestingly, human genetic defects of a biosynthetic enzyme, D4ST-1, results in a type of Ehlers-Danlos syndrome and these patients have aberrant assembly of the collagen fibrils (7). The impaired collagen assembly is most likely a result of reduced IdoA-content in decorin present in these patients (8).

TGF-beta (transforming growth factor-beta) activated fibroblasts demonstrated reduced levels of epimerase activity, an effect that was further reduced by the combinatory effect of TGF-beta, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (9). In another study, PDGF promoted migration of fibroblasts. The mechanism was proposed to involve upregulation of IdoA in the proteoglycan CD44 (10).

Different proteoglycans produced by the same cell can vary greatly in their IdoA content. Decorin and biglycan, for instance, have been found to contain blocks of IdoA whereas versican only has isolated IdoA residues. One study suggested that the core protein regulates the activity of the DS-epimerases. This was demonstrated by generation of chimeric proteins of decorin and CSF, a part-time proteoglycan. The chimeric decorin contained less IdoA compared to the unmodified decorin (11). This suggests that core proteins carry information, influencing epimerization.

Sulfation

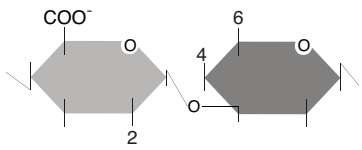
The sulfation of CS/DS is catalyzed by sulfotransferases using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfate donor. These modifications occur at three positions in the disaccharide; GalNAc is sulfated at position C4 and C6 while GlcA/IdoA is sulfated at position C2 (12) (figure 3).

The O-sulfotransferases (O-ST) acting on CS/DS chains have eight members that fall into three categorizes, 4-O-STs, 6-O-STs, and 2-O-STs. 4-O-STs consist of C4ST-1, C4ST-2, and C4ST-3 and they transfer sulfate with slightly different preferences. C4ST-1 has a predisposition for GalNAc flanked by two GlcAs, whereas C4ST-2 acts on GalNAc flanked by either GlcA or IdoA and has lower specific activity than C4ST-1 (13). C4ST-3 is less well characterized but acts both on desulfated chondroitin and dermatan (14). The last member of the 4-O-ST family is dermatan 4-O-sulfotransferase (D4ST-1), which sulfates GalNAc flanked by two IdoA residues.

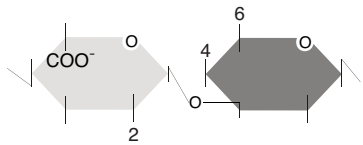
The 6-O-ST family contains two sulfotransferases, C6ST-1 (15,16) and C6ST-2 (17). C6ST-1 has been shown with loss-of-function models to be the major enzyme *in vivo* (18) and mutations in human are known to result in severe chondrodysplasia (19). Two additional enzymes add sulfate groups on monosulfated disaccharides, i.e. N-acetylgalactosamine 4-sulfate 6-O-ST (GalNAc4ST-6ST) (20) and uronosyl 2-O-ST (CS2ST/UST) (21). GalNAc4ST-6ST transfers a sulfate group to position 6 on a 4-O-sulfated GalNAc. 2-O-sulfation on GlcA/IdoA adjacent to a GalNAc that has already been 4- or 6-sulfated is catalyzed by CS2ST/UST. In summary, CS/DS chains can be modified considerably and the diversity of the chain is reflected in its many biological properties.

Composition of CS/DS structures

The sulfations catalyzed by sulfotransferases result in CS/DS structures depicted in figure 3. The CS/DS chain mainly contains monosulfated disaccharides. These disaccharides are composed of a single sulfated GalNAc residue flanked by GlcA/IdoA. If GalNAc is next to an IdoA unit, the GalNAc residue is predominately 4-O-sulfated (iA unit), whereas a small proportion are disulfated, i.e. have an extra 2-O-sulfation at the IdoA residue (iB unit). In mammals, two additional disulfated structures are present. The E/iE units are implicated in many physiological events such as growth factor binding and migration (22). When GlcA is 2-O-sulfated and situated next to a GalNAc that is either 4- or 6-O-sulfated, the structures are termed B and D units, respectively. When they contain IdoA instead of GlcA, the structures are named iB and iD.



GlcA-GalNAc



IdoA-GalNAc

Sulfation position	Nomenclature
No sulfation	O unit
4	A unit
2,4	B unit
6	C unit
2,6	D unit
4,6	E unit

Sulfation position	Nomenclature
No sulfation	iO unit
4	iA unit
2,4	iB unit
6	iC unit
2,6	iD unit
4,6	iE unit

Figure 3. Disaccharide units present in mammalian CS/DS. Disaccharide structures found in CS/DS generated by sulfation and epimerization. The coding system is depicted by a letter code which represent the sulfation pattern whereas iduronic acid is indicated by 'i'.

CS/DS has been purified mainly from marine animals and mammals. CS/DS contains a mixture of disaccharide units with preference for certain units. For instance, CS-A mainly contains GlcA flanked by GalNAc 4-O-sulfated structures, whereas CS-B and CS-E contain relative high amounts of iB and E units, respectively. However, CS/DS chain is never composed of a single repeating disaccharide unit.

Tumor development

Cancer is a group of diseases, consisting of unregulated cell growth and invasion of cancer cells in nearby and distant organs, leading to disturbed physiology. Tumor cells are surrounded by blood vessels, inflammatory cells, and fibroblasts. Cancers have long been considered to be unhealed wounds, where the cancer cells recruit blood vessels and stroma cells through production of growth factors and cytokines (23).

Cancer cells gain a number of characteristic features necessary for tumor development such as cell death resistance, sustained proliferative signals, and growth suppression evasion. Increased proliferation can be accomplished in number of ways, for example by increased density of growth factor receptors, altered growth factor response, or upregulated production of growth factors (24). In malignant melanoma, mutation of the B-Raf protein results in continuous signaling of the MAP-kinase pathway, resulting in enhanced proliferation (25). Tumor cells evade apoptosis by several mechanisms such as retinoblastoma (RB)-associated mutations and tumor suppressor TP53 mutations (26). Telomerase, a DNA polymerase, is virtually absent from normal cells whereas tumor cells acquire the enzyme and thereby become resistant to apoptosis and senescence (27). Changes in energy metabolism were first described by Warburg when he observed that tumor cells alter their metabolic needs by using anaerobic glycolysis as their major energy-supplying pathway. Cancer cells compensate for the lower ATP yield by upregulation of glucose transporters (28). Historically, tumors have been considered to be homogeneous cell populations, but during the last two decades a subpopulation of cancer cells named cancer stem cells has attracted attention. These cells have transcriptional profiles in common with normal tissue stem cells. Cancer stem cells are thought to have highly potent tumorigenic properties (29). Inflammation, another cancer-promoting process, is present in all tumors. The inflammatory process modifies the microenvironment and causes the release of growth factors (30). For example inflammation upregulates heparanase, an enzyme that cleaves HS, resulting in increased release of tumor-promoting growth factors (31).

Thus, cancer development is an intricate process involving numerous pathways. PGs are present either at the cell surface or in the ECM. They are known to participate in tumor-regulatory events such as angiogenesis, metastasis, and tumor growth and will be discussed later in this overview.

Tumor stroma

Interaction of tumor and stromal cells

The ECM has traditionally been regarded merely as a supporting tissue, however emerging data supports the role of ECM in tumor growth and progression. Formation of cancer stroma, which is a specialized cancer-initiated ECM, requires crosstalk between tumor cells and stromal cells (32). Fibroblasts, endothelial, inflammatory, and immune cells are different types of stromal, which produce and modify the stroma. Cancer stroma varies greatly between different tumor forms, both in terms of composition and quantity. ECM structural proteins such as collagens, laminins, proteoglycans, and hyaluronan are mainly produced by fibroblasts. In addition, fibroblasts secrete a number of cancer-promoting growth factors. An elegant study by Kuperwasser et al. showed that human tumor-derived stromal fibroblasts are necessary for the establishment of human breast tumor xenografts (33).

Tumor cells modulate the stromal compartment by production of growth factors such as vascular endothelial growth factor (VEGF), FGF, PDGF, and TGF-beta. This generates a wound-like microenvironment (34) with increased angiogenesis (35) and an increased inflammatory response (36). Angiogenesis is the formation of vessels from the pre-existing vascular network, which is necessary for tumor development. The pro-angiogenic drive of a cancer results in blood vessels with different morphology—i.e. unorganized, distorted, leaky, and hemorrhagic vessels, as a result of an unbalanced regulation of pro- and anti-angiogenic factors, for example VEGF (37,38). The HS chain of HS-PGs can bind VEGF and thus modulate angiogenesis (39,40). Recently, CS/DS has been shown to rescue VEGF-mediated angiogenesis in the absence of HS (41). In addition to VEGF expression, the angiogenic drive in tumor tissues is governed by the degree of hypoxia (42).

PDGFs are paracrine mitogens that activate fibroblasts, smooth-muscle cells, and immune cells during wound healing (43). In particular, PDGFs have essential roles in the formation of new blood vessels (44). PDGF secreted by melanoma cells induces pericyte proliferation and recruitment (45), and increased enhanced angiogenesis was seen in xenografts of PDGF-overexpressing melanoma cells (46).

During inflammation, fibroblasts, adipocytes (47), and smooth-muscle cells (36) upregulate their production of matrix metalloproteinases (MMPs) and growth factors. Increased production of MMPs by cancer and stromal cells results in degradation of the ECM and basement membrane (48). Upon degradation, the ECM-sequestered growth factors

and protein fragments are released, promoting tumorigenesis (48,49). In a co-culture study, benign and malignant skin carcinoma cells were cultured with stromal fibroblasts. Unlike benign tumor cells, malignant tumor cells had increased production of MMP1 and MMP9 when co-cultured with stromal fibroblasts, demonstrating the active crosstalk between malignant tumor cells and stromal fibroblasts (50).

Tumor-modulating growth factors

Fibroblasts have been shown to be modifiers of cancer, and a certain subpopulation of fibroblasts (i.e. cancer-associated fibroblasts) is particularly important. Cancer-associated fibroblasts (CAFs) secrete a number of growth factors and cytokines that promote tumorigenesis. Some of these growth factors are prototypic epithelial mitogens such as FGFs, TGF-beta, and HGF. HS has been found to interact with several of these growth factors (51). TGF-beta has dual roles in cancerogenesis where opposing functions of TGF-beta as a tumor suppressor and a promotor of metastasis has been described (52). In support of the latter, TGF-beta influences metastasis by the disruption of adherens junctions and induction of migration (53). Moreover, plasma TGF-beta is a predictor of early metastasis (54).

FGFs and their receptors (FGFRs) are often mutated in human tumors (55). Prostate homeostasis is partially regulated by the expression of FGFs, which is altered during neoplastic transformation. FGF1, FGF2, and FGF6-10 are produced by stromal cells and promote proliferation of epithelial cells in paracrine manner (56). It is noteworthy that mesenchymal overexpression of FGF10 leads to formation of prostate adenocarcinoma *in vivo* (57).

HGF and its receptor, MET, are tumor-promoting factors involved in processes such as metastasis and cell motility (58). Upon binding of HGF, the MET receptor dimerizes and elicits signaling cascades. This signaling transduction pathway results in activation of several kinases such as MAPK, ERK1/2, JNKs, p38, PI3K-Akt, STATs, and NF-kB (58). CAFs are known to overexpress HGF (59) and to stimulate cancer cells in a paracrine way. Inflammatory cytokines and growth factors in the neoplastic stroma induce the upregulation of the MET receptor on tumor cells of epithelial origin (60). The MET receptor is also overexpressed in ESCC (61). High levels of HGF and MET receptor correlates to poor prognosis (62). Furthermore, MET expression has been associated with metastasis.

Cell migration

Migration is necessary in many physiological events such as implantation, embryogenesis, and during several pathological conditions. An improved understanding of this could lead to new therapeutic avenues for e.g. cancer, rheumatoid arthritis, and multiple sclerosis (63). In response to pro-migratory stimuli, the cell forms protrusions. These can either be lamellipodia, filopodia, blebs, or invadopodia. *In vivo* lamellipodia facilitate long-distance movement whereas filopodia sense the ECM to form focal adhesions. Membrane blebbing has been suggested to participate in the migration of cells during development. Invadopodia have been implicated in cell invasion and metastasis. The protrusions are regulated by Rho GTPases, membrane phospholipids, and other signaling molecules. Furthermore, filopodia and lamellipodia are promoted by polymerization of actin, which pushes the membrane forward (64).

Lamellipodia have been described in several cell types such as fibroblasts, muscle precursor cells, and epithelial cells (65,66). In the lamellipodium, the Arp2/3 complex forms an actin network by binding to the sides of the actin filaments. A number of proteins such as profilin regulate the organization and polymerization of actin by binding and sequestering actin monomers. Furthermore, elongation is stopped by capping factors, thus redirecting the polymerization to new actin threads. The ADF/cofilin family disassembles filaments, which increases the pool of actin monomers available. Filamin A and α-actin crosslink actin filaments into a network and anchor the actin cytoskeleton to membrane proteins (67).

While lamellipodia are composed of a branched network, filopodial protrusions are generated by a treadmilling mechanism. Filopodia act as sensors for inspection of the surrounding environment. Filopodia have been suggested either to be an independent actin polymerization process mediated by formins, or to emerge from lamellipodia (68). In filopodia, actin filaments are produced by release of actin at the rear end and polymerization at the opposite end. ENA/VASP proteins inhibit capping and branching, thereby facilitating elongation and stiffness necessary to drive the plasma membrane of filipodia forward.

Invadopodia have been implicated in invasion and metastasis, since they participate in degradation of the ECM. The major feature that differentiates invadopodia from filopodia and lamellipodia is the expression of matrix-degrading proteases such as membrane type-1 metalloprotease (MT1-MMP) (69).

The Rho family of proteins control the formation of actin and adhesion complexes, and therefore the generation of lamellipodia and filopodia. RAC, Cdc42, and RhoA, are small guanosine triphosphate binding proteins (GTPases) belonging to the Rho family (70). Cdc42

facilitates cell polarity, i.e. different molecular processes at the front and at the back of a migrating cell (71). Cdc42 influences polarity by re-localization of the microtubule-organizing center and microtubule production. In the lamellipodia, RAC induces branching of the actin web through a signaling cascade that activates the Arp2/3 complex (72). RhoA participates in tail retraction (73). RhoA levels must be carefully tuned, since both ablation (74) and upregulation of RhoA leads to loss of lamellipodia.

Lamellipodia, filopodia, and invadopodia anchor to the ECM by adhesion complexes. The adhesion complexes are composed of over 150 different molecules (75). Integrins, cell membrane-bound receptors, facilitate the interaction between the ECM and the cytosolic actin-linking proteins. Cytosolic complex is associated with signaling and adapter proteins such as focal adhesion kinase (FAK), vinculin, and paxillin (76). On activation of integrins, FAK assembles and disassembles the complex that links integrin to the actin cytoskeleton. Thus, FAK regulates cell migration (77). FAK-deficient cells have a higher number of focal adhesion contacts and migrate less than normal fibroblasts (78). Several studies have shown a correlation between FAK expression and cancer development (79). During cell invasion, FAK expression has been found to both induce and reduce migration. Cancer cells have lower FAK expression and activation during EGF-mediated invasion (80). However, downregulation of FAK reduces cytotrophoblast invasion (81).

Interestingly, CS/DS is known to be involved in migration as enzymatic removal of the chain decreases migratory activity of both macrophages and osteosarcoma cells (82,83).

Metastasis

Metastatic lesions are signs of spread of disease and poor prognosis. Treatments such as surgery, chemotherapy, and radiotherapy are more likely to succeed when no metastases have been formed. The metastatic process can be divided into two major events: invasion of the ECM and vascular dissemination, followed by colonization at a distant site (84). It is a highly inefficient process since millions of cells may enter the vascular system every day and eventually only a few macroscopic metastases might develop (85,86). Invasion of the ECM consists of a series of steps such as loss of cell-cell interactions, degradation of the ECM, and tumor cell invasion. A cancer cell disrupts its E-cadherin-dependent junctions (87) and degrades the ECM by production of MMPs. Subsequently, the tumor cell continuously assembles and disassembles its focal adhesion complexes to facilitate migration along collagen fibers (88).

Integrins and tumor cell receptors, such as cell-surface proteoglycans, mediate anchor points in the ECM and facilitate actin re-organization signals. Chemokine and growth factor gradients promote migration of cancer cells, e.g. tumor-associated macrophages generate EGF gradients (89). These macrophages are located in the tumor margin or close to blood vessels. Thus, they facilitate tumor cell invasion and intravasation (90). Accordingly, overexpression of the EGF receptor on cancer cells enhances motility and increases the likelihood of forming metastases (91).

When cancer cells enter the bloodstream, the environmental conditions change drastically, i.e. absence of substratum, the presence of immune cells, and velocity-induced shear forces. Tumor cells form emboli, aggregate with platelets, to evade elimination by the immune system, a process partially mediated by P-selectin (92,93). In addition, emboli contribute to metastatic formation and arrest of blood flow in distant organs (94). Many tumors derived from the gastrointestinal tract form metastases in the liver, whereas breast and prostate cancer can lead to bone metastases. Metastatic colonization of distant organs is both dependent on the anatomical location and the microenvironment. Experimental studies have indicated that osteopontin (95) and stromal-derived factor-1 (96) form a microenvironment that is prone to develop metastatic lesions.

Thus, the metastatic process is determined by a number events occurring at the primary site, in the bloodstream, and at the distant organ. Several CS/DS-PGs such as CSPG4/NG2 and CD44 are known to participate in this process.

Squamous cell carcinoma of the esophagus

Esophageal cancer is a severe and lethal disease, and globally it is the seventh and fifth most common tumor in females and males, respectively (97). Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma are the two major types of esophageal cancer. Ninety-five percent of all esophageal cancers worldwide are ESCCs, although adenocarcinoma is the most rapidly increasing tumor form in some parts of the western world. In the United States, the incidence of adenocarcinoma has surpassed that of ESCC. The 3-year survival rate for ESCC is 15-20% in the United States (98). There is a strong correlation between alcohol consumption and smoking and the development of ESCC (99). Furthermore, ESCC is associated with low socioeconomic status, malnutrition, liver dysfunction, intake of nitrosamines (97), and pulmonary co-morbidities (100). North central China has a particularly high prevalence of ESCC, which has been suggested to be related to genetic factors (101). The tumor is often diagnosed after the age of 60,

and the first symptom is usually dysphagia. Weight loss is a symptom of progressive disease and 75% of all patients have lymph node involvement at the time of diagnosis. The primary examination is an esophago-gastroduodenoscopy (EGD) in combination with a histopathological diagnosis. A complete examination includes endoscopic ultrasound and a computed tomography (CT) scan of the chest and abdomen to detect potentially distant metastases. Fluorodeoxyglucose-positron emission tomography (FDG-PET)/CT is a more sensitive method for detection of metastatic lesions. It is occasionally used to follow therapeutic responses (99). ESCC is classified according to TNM i.e. tumor invasiveness (T), lymph node engagement (N), and the presence of metastases (M). Esophagectomy is the primary treatment, often in combination with chemotherapy and radiotherapy or chemotherapy alone, which further increases the survival time (102).

ESCCs gain many of the genetic alterations commonly associated with neoplastic development. These mutations cause dysregulation of proteins involved in processes such as signal transduction, apoptosis, transcriptional regulation, and cell cycle control (103). The tobacco-induced cancer gene TP53 is frequently inactivated in ESCC, often combined with k-ras mutations and loss of retinoic acid receptor (104). In ESCC, signal transduction molecules such as FAK, HGF, ERK-1/2, STAT3, and particularly EGFR have been implicated in disease development. EGFR is associated with cancer invasion and has been suggested to be a prognostic factor (105). HGF expression increases both in tissue (106) and in plasma derived from ESCC. Furthermore, high levels of serum HGF correlate with poor long-term survival (107). A recent report has shown the importance of HGF for ESCC cell-mediated invasion in a 3D cell culture system (108). Since ESCC is usually detected at an advanced stage, it has been considered particularly important to identify new cancer markers. Correlations have been found between cyclin D1 (109), p53 (110), and E-cadherin (111) levels and survival rate and disease progression, although none of them have been sufficiently specific to be put into clinical use.

Biological functions of CS/DS

CS/DS structures in cancer

GAGs have been implicated in cancer progression and metastasis. In cancer, the sulfation pattern of CS/DS is different from that of normal tissue. 6-O-sulfated monosulfated disaccharides are increased in cancer compared to normal tissues whereas 4-O-sulfated monosulfated disaccharides are decreased (112). Moreover, the E units are highly expressed in ovarian adenocarcinomas and mediate VEGF binding (113) (figure 4A). During metastasis of tumor cells, E units expressed on the cell surface mediate colonization of the lung and liver (114,115). This process has been proposed to be mediated by the receptor RAGE, which is mainly expressed in the lungs (116). Another plausible explanation may be that E units mediate platelet binding, which results in the formation of tumor microemboli. These microemboli protect tumor cells against elimination by the immune system. P-selectin expressed on platelets mediates interactions between cancer cells and platelets. In a number of studies, CS/DS has been shown to bind to P-selectin (figure 4B). For example, CS-E and CS-B have been found to inhibit the binding of recombinant P-selectin to breast cancer cells (117). Moreover, two CS/DS structures composed of iB units or iD units isolated from two marine animals have been found to inhibit metastasis in a P-selectin-dependent manner in a metastatic tumor model. This was demonstrated by injection of the CS/DS structures in WT and P-selectin-deficient mice (118).

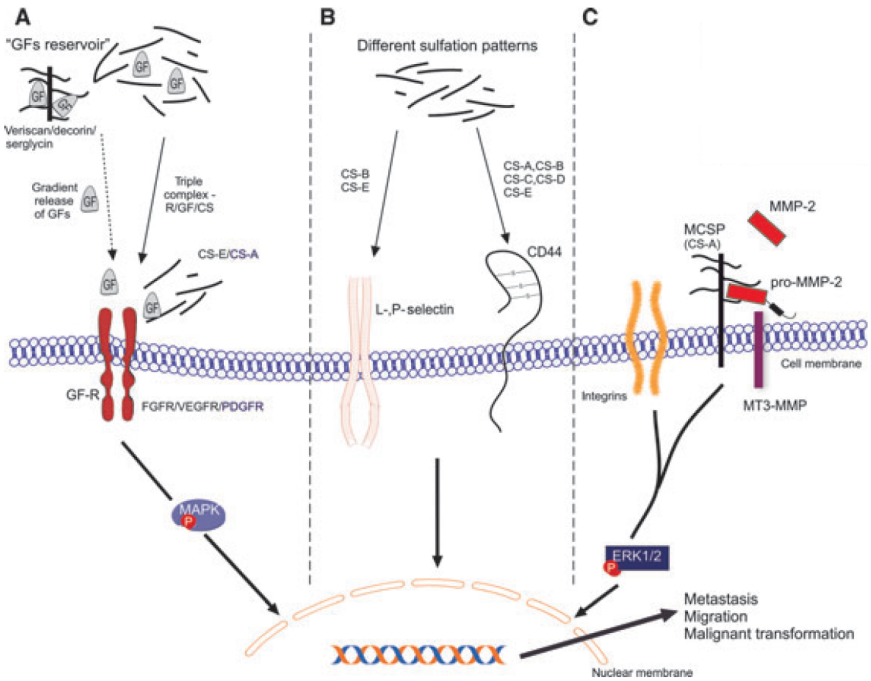


Figure 4. Involvement of CS/DS structures and CS/DS-PGs in receptor signaling and cancer-related processes. *A,* Growth factor sequestering and presentation to signaling receptors. *B,* Interaction with L- and P-selectins and CD44. *C,* Activation of MMP2. Adapted from (119).

Several reports have shown that CS/DS participates in growth factor and chemokine binding. For example, IdoA is required for HGF-mediated binding and a tetrasaccharide has been found to be the minimum structure to confer affinity (120). CS-E has been shown to interact with several of the members of the FGF family. Consequently, CS-E may act as co-receptor to regulate and present growth factors (121) (figure 4A). In addition, CS-A and PDGF-BB have co-stimulatory effects on tyrosine kinase pathways, enhancing *in vitro* migration of fibrosarcoma cells (122). Exogenously added CS-B has been found to inhibit the proliferation of normal and malignant cells (123). Removal of CS/DS chains by chondroitinase B has also been shown to inhibit the migration and invasion of tumor cells (124).

Binding of metalloproteinases (MMPs) is another cancer-promoting feature in which CS/DS chains regulate activity. MMPs are essential for degradation of the extracellular matrix and cancer invasion

(119). CS-A binds to both pro-MMP-2 and MT3-MMP and this leads to activation of MMP2 (125) (figure 4C). In conclusion, CS/DS has been implicated in several biological processes and disulfated disaccharides in particular are mediators of many functions.

CS/DS proteoglycans in cancer

A cancer tissue modifies the ECM to favor tumor growth (126). Proteoglycans are key components of the ECM that regulate a number of cellular features, such as proliferation, migration, and invasion. The amounts of PGs and GAGs are increased in cancer stroma relative to non-neoplastic tissue, and they are even more concentrated in the growing edge of the tumor (127). As previously described, reciprocal signals between fibroblasts and cancer cells trigger induction of cancer-associated fibroblasts (CAFs). CAFs produce ECM, which promotes cancer development. CS/DS-PGs can influence the properties of cancer stroma. CS/DS-PGs are divided into the following groups: basement membrane PGs, nervous system PGs, extracellular PGs, cell-surface PGs, other PGs, and collagens (Table I).

Table 1. CS/DS PGs implicated in tumorigenesis and functions of the CS/DS chain

CS/DS Proteoglycans	IdoA	Functions in tumor development	Functions of CS/DS and protein bound to CS/DS
Basement membrane or basement membrane connected CS/DS-PGs			
Perlecan	N.A.	Regulator of angiogenesis	N.A.
Bamacan	N.A.	Anchorage-independent growth	N.A.
Leprecan	N.A.	N.A.	N.A.
Collagen XV	N.A.	Suppresses tumor growth	N.A.
Nervous system			
Neurocan	N.A.	Upregulated in astrocytoma	N-CAM, HB-GAM, amphoterin
Phosphacan (Receptor Protein Tyrosine Phosphatase β/ζ)	+	Mediates migration and adhesion	HB-GAM, amphoterin, midkine
Brevican	N.A.	Proteolytically cleaved brevican promotes glioma invasion	neuritogenic activity
Appican (AβPPisofom)	N.A.	N.A.	midkine, pleiotrophin
Neuroglycan C	N.A.	N.A.	N.A.
Extracellular space			
Aggrecan	-	Stage-related loss in laryngeal squamous cell carcinoma	water retention
Versican	+	Increases motility, proliferation and metastasis	FGF family, L- and P-selectin, chemokines
Decorin	+	Suppresses proliferation, cell migration and angiogenesis	FGF2, FGF7, HGF, HCII, VWF, α2β1, tenascin-X, fibril formation
Biglycan	+	Upregulated in gastric cancer and expressed by tumor endothelial cell	HCII, FGF family
Epiphycan	+	N.A.	N.A.

Cell surface			
CSPG4 (NG2/MCSP)	N.A.	Regulates tumor cell growth, cell motility and associated with patient survival	N.A.
Betaglycan	N.A.	Suppresses cancer progression and metastasis	N.A.
Syndecan-1	+	Regulate tumor cell survival and proliferation	midkine, pleiotrophin, FGF7
Syndecan-3	N.A.	Expressed in tumor stromal vessels	N.A.
Syndecan-4	N.A.	Upregulated in cancer and mediates cell spreading	midkine, pleiotrophin
CD44	+	Tumor growth, angiogenesis, metastasis	migration
Neuropilin-1	N.A.	Tumor growth, angiogenesis, metastasis	VEGF signalling
α5β1 integrin	N.A.	Influences tumor growth and angiogenesis	N.A.
Others			
SRPX2	+	Overexpressed in gastrointestinal cancer and increases endothelial proliferation	HGF
Serglycin	N.A.	Promotes metastasis	maturation of granules
Endocan	+	Promotes tumor formation and expression correlates to survival rate	HGF
Testican-1	N.A.	N.A.	N.A.
Testican-2	N.A.	Promotes invasion	N.A.
Testican-3	N.A.	Inhibits invasion	N.A.
Collagens			
Collagen IX	N.A.	N.A.	N.A.
Collagen XII	N.A.	Highly expressed by cancer-associated fibroblasts in colon cancer	N.A.
Collagen XIV	N.A.	N.A.	N.A.

N.A.: not analyzed

Extracellular PGs

Of the ECM PGs, the cancer-attenuating PG decorin is the most extensively studied. Antiproliferative effects have been demonstrated through stable transfection of decorin in CHO cells (128). The mechanism has been suggested to involve TGF-beta sequestration by the core protein of decorin (129). Intriguingly, overexpression of decorin suppresses malignant progression by induction of the cyclin-dependent kinase inhibitor p21 (130). Data accumulated over the last few decades suggest that decorin interacts with two essential growth-promoting receptors, the EGF receptor and MET receptor (131). Decorin binds to the EGF receptor and causes its internalization (132), thereby suppressing tumor growth (133,134). Decorin antagonistically targets the MET receptor and attenuates the signaling pathway (135). It is a beta-catenin-mediated suppression, which prevents cancer cell invasion and migration (136). None of the anti-tumorgenic properties of decorin are known to be mediated by the CS/DS chain. Moreover, the cancer-attenuating effect is also mediated by decorin induced expression of proinflammatory molecules such as programmed cell death 4 (PDCD4) (137).

Versican—another ECM PG—participates in adhesion, proliferation, migration, and angiogenesis (138). Versican expression initiates formation of pulmonary adenocarcinoma. Moreover, downregulation of versican results in reduced tumor growth *in vivo* (139). It has been suggested that cells incorporate versican and HA into their pericellular matrix to promote migration (140). Incorporated versican and HA are visible at the trailing end of migrating cells, while no HA or versican is detectable at the leading edge of the cell. Versican and HA are thought to inhibit CD44-mediated ECM binding; hence the detachment at the trailing end. Recently, a study showed that versican accelerates the formation of lung metastases (141).

In addition, endocan, biglycan, testicans, and SRPX2 contribute to tumor formation. Endocan is reported to be a tumor endothelial marker in non-small cell lung cancer (142), hepatocarcinoma (143), and kidney cancer (144). There is a correlation between endocan expression, in blood and tissue, and poor survival in non-small cell lung cancer and hepatocarcinoma, respectively. The CS/DS chain of endocan binds to HGF, and this interaction is probably due to the large amount of IdoA in the CS/DS chain (145). Accordingly, endocan enhances HGF-mediated proliferation (146). Endocan-overexpressing cells were found to form tumors while control cells failed to establish xenogeneic tumors. Even more important, cells lacking the CS/DS chain could not generate tumors (147).

Biglycan is also described as a tumor endothelial marker. Downregulation of biglycan inhibits tube formation in tumor-derived

endothelial cells (148). Additionally, biglycan is known to be overexpressed in tumor tissues (149,150). The testican family has dual functions in response to MMP regulation. Testican-3 promotes MMP-mediated invasion while testican-2 abrogates this function. A recently described CS/DS-PG, SRPX2, induces endothelial proliferation in a HGF-dependent manner (151).

Cell-surface PGs

Several cell membrane-bound CS/DS-PGs such as CSPG4, CD44, neuropilin-1, and betaglycan have been thoroughly investigated. Surprisingly, the presence of IdoA is disregarded in most cases. CSPG4, also called NG2 or MCSP, is associated with cancer development, angiogenesis, and tumor progression. CSPG4 activates receptor tyrosine kinases, integrins, and FAK. These signaling transduction molecules affect multiple cellular functions such as migration, epithelial-mesenchymal transition, tumor growth, survival, and re-organization of the cytoskeleton (152). Constitutive CSPG4 expression results in increased activation of the MET receptor whereas loss of MET expression restricts CSPG4-mediated growth and migration (153). CD44, a PG with both HS or CS/DS chains, is implicated in many of the same functions as CSPG4. However, CD44 is a major receptor for hyaluronic acid (HA), which is the a major component of the ECM (154). The extracellular interaction is essential for HA-dependent migration whereas the cytoplasmic tail of CD44 binds actin cytoskeleton-related proteins (155). CD44 is located in lamellipodia and guides migration (156). One study found that a CD44-blocking antibody inhibited lamellipodia formation in an epithelial cell line (157). MET is also a partner for CD44 since a CD44 isoform, CD44v3, is known to promote phosphorylation of MET through the heparan sulfate chain (158). However, it is unclear whether the HS chain mediates this effect, since another study has suggested it to be dependent on the CD44v6 isoform (159). Thus, HGF, MET, and CD44 form a complex that activates MET (159). The cytoplasmic domain of CD44 is responsible for assembly of a complex between the intracellular domain of MET and a number of molecules including GRB2 and the proteins F-actin and moesin. Also, MET-mediated activation of Ras requires the cytoplasmic domain of CD44 (160).

CS/DS structures in neuritogenesis

CS/DS structures are implicated in brain development (161) and CNS injury (162). The CS/DS bioenzymatic machinery is carefully regulated during brain development. IdoA-containing structures (iA and iB) are mainly expressed in the cerebellum, and IdoA content increases during postnatal brain development (163). In situ hybridization suggests that DS-epi2 is ubiquitously expressed during postnatal brain development. Moreover, mRNA expression analysis has shown that expression of DS-epi2 is higher than expression of DS-epi1 (164). Using an antibody to the iD unit, the distribution of iD units in developing mouse brain was mapped. In the cerebellum, the iD unit was detectable on postnatal day 7 but was absent in adult brain. On the contrary, in the hippocampus the iD unit was expressed in adult brain (165). CS/DS purified from embryonic pig brain was found to be composed of 8–9% IdoA while the adult brain contained 1-2% of IdoA. (166). Interestingly, the embryo-derived CS/DS shows enhanced binding of FGFs (FGF-2, -10, and -18), pleiotrophin, and midkine. This binding is abolished when the CS/DS are pretreated with chondroitinase B, which clearly indicates the importance of IdoA. Hippocampal neurons were plated on CS/DS derived either from embryonic brain or adult brain. On the IdoA-rich substrate obtained from embryonic brain, neurons were activated—which contrasted with the results with neurons plated on adult brain CS/DS (166). The iB unit and E unit are believed to facilitate binding of a number of growth factors such as pleiotrophin, midkine, VEGF, FGF-2, FGF-7, and HGF. These binding properties are eliminated by chondroitinase ABC and B, but chondroitinase AC-I and AC-II only partially reduce the binding activity. Moreover, CS/DS derived from marine organisms from CS/DS chains rich in IdoA-containing disulfated disaccharides stimulate neurite outgrowth (167).

CS/DS proteoglycans in neuritogenesis

CS/DS-PGs are the most abundant PGs in the CNS, and they have important roles in regulating the microenvironment. During brain development, CS/DS-PGs are spatio-temporally regulated. The lectican/hyalectan family has four members (aggrecan, versican, neurocan, and brevican) that are involved in a variety of functions. During development of chick brain, aggrecan blocks neural crest migration—in contrast to versican, which stimulates migration (168). Neurocan, a CNS-specific PG, binds several adhesion molecules such as N-CAM and Ng-CAM/L1. This interaction inhibits neural attachment and outgrowth in vitro (169). During postnatal brain development in the rat, brevican expression progressively increases and reaches a plateau in the adult brain (170).

A number of transmembrane part-time CS/DS-PGs are expressed in brain such as neuropilin-1, CSPG4, and phosphacan (i.e. receptor-type protein tyrosine phosphatase ζ). Phosphacan mediates cell adhesion (171) and growth factor binding (172,173), and it controls neuron growth (169). CSPG4 is mainly expressed by oligodendroglial precursor cells and pericytes in the developing CNS. CSPG4 is detected during embryonic development, peaks in the postnatal period (P8-P12), and subsequently declines. The role of CSPG4 in regulation of neurite growth is debated. In one study, it was found that immunopurified CSPG4 inhibits growth whereas another study indicated that CSPG4 may have dual regulatory functions. Neuropilin-1 is a co-receptor for semaphorin-3A, which acts as an axon repellent factor (174). Appican and testicans are secreted CS/DS-PGs, and both are present in the postnatal mammalian brain. Appican is the PG form the amyloid precursor protein and the CS/DS chain of appican consists of appreciable amounts of E units, which facilitate growth factor binding (175,176). The testican family was discovered only recently, and the core protein has a calcium-binding domain. Testican-1 is expressed in pyramidal neurons of the mouse hippocampus (177).

The present investigation

Aim

The presence of IdoA in CS/DS has been well known for a long time, although the biosynthetic enzymes responsible for its synthesis, DS-epi1 and DS-epi2, were only recently identified and cloned (178,179). In the literature, biological functions for IdoA in CS/DS are mainly reported in *in vitro* studies. In these reports, exogenous CS/DS structures were used in cell culture and growth factor binding experiments. Our studies are the first to use genetic models to identify biological functions for DS-epi1 and DS-epi2 in tumor cell function and brain development, respectively. The overall aim of this thesis was to investigate the biological roles of IdoA in CS/DS.

Methodology

The following methods have been used in this thesis

Aortic ring assay	*Ex vivo* angiogenesis model
Confocal laser scanning microscopy	High resolution microscopy with depth selectivity
Chromatography	Method to separate components of a mixture
Epifluorescence microscopy	Optical microscopy using fluorescence to observe the specimen
Epimerase activity assay	Measure released ^{3}H from ^{3}H-labeled K4 polysaccharides
Flow cytometry	Laser based single-cell analysis
Generation of genetically engineered mice	Disruption of an existing gene by substitution with an artificial DNA piece
Immunoblot assays	Gel electrophoretic separation of proteins for antibody detection
Immunohistochemistry	Antibody based detection of antigens in a tissue
In vitro migration assays	Cell migration analysis using scratch wound assay or transwell pore migration system
In vitro invasion assays	Cell invasion analysis using Matrigel coated transwell pore migration system
In vitro proliferation assay	Crystal violet based proliferation assay
In vivo synegenic tumor model	Subcutaneous inoculation of tumor cells into mice
Mass spectrometry analysis	Technique measuring mass-to-charge ratio of charged particles
Metabolic labeling and isolation of PGs and GAGs	Radioactive labeling of GAGs followed by anion-exchange chromatography
Microarray analysis	Chip based mRNA gene expression array
Phosphokinase array	Protein array which determines phosphorylated proteins
q-RT-PCR	Real-time polymerases chain reaction based quantitative measurement of mRNA
RNA interference	Technique which induces gene silencing
Sulfotransferase activity assay	Measure radioactive incorporation of ^{35}S in the GAG chain

Paper I

Dermatan sulfate is involved in tumorigenic properties of esophagus squamous cell carcinoma

DS-epi1, the enzyme responsible for the conversion of IdoA in CS/DS, was cloned with unknown function and named SART2 (180). DS-epi1 was proposed to be a tumor-specific antigen. After identification of the enzymatic activity of DS-epi1, we determined that DS-epi1 was ubiquitously present in normal tissue. As DS-epi1 was highly expressed in cancer, we hypothesized that IdoA may be involved in tumor development.

Results

We found DS epimerase activity and DS epimerase 1 expression to be elevated in patient material from ESCC compared to normal tissues. In biopsies, stromal- and cancer cells were found to express DS-epi1, as shown by immunohistochemistry. To understand the function of IdoA, we stably downregulated DS-epi1 in ESCC cell lines using shRNA sequences.

Traditionally, reports of IdoA have focused on ECM PGs such as biglycan, decorin, and versican. Here we investigated the presence and functions of cell-surface-bound IdoA. Previous reports have demonstrated that IdoA-containing structures of CS/DS bind to HGF (181). Whereas these studies made use of exogenous CS/DS of defined structure and recombinant HGF, we investigated this interaction in a biological context using genetic models.

We found that IdoA promotes binding of HGF to its receptor and, more importantly, was essential for MET-dependent signaling. Moreover, IdoA is involved in migration and invasion. As MET is involved in both cytoskeletal and adhesion complexes, we hypothesized that IdoA influences these structures. Indeed, migrating DS-epi1-downregulated cells in the presence of HGF showed a disassembled actin organization, i.e. fewer cytoplasmic stress fibers and fewer plasma membrane protrusions than control cells. Focal adhesion complexes of DS-epi1-downregulated cells were homogeneously distributed at the cell membrane, whereas the focal adhesions complexes of control cells were primarily located at the leading edge. Thus, cell-surface IdoA might both function as a co-receptor for HGF, and be involved in migration and invasion of cancer cells.

In accordance with previous studies, we found a 5-fold increase in CS/DS in tumor tissue compared to normal tissue. These data were

accompanied by upregulation of 4-O- and 6-O-sulfotransferase activities. Despite the increased epimerase activity, the total amount of IdoA in cancer tissue and normal tissue was unchanged, indicating a reduced number of IdoA residues per chain.

In summary, we found IdoA to be important in cell migration, invasion and HGF-mediated cell signaling of cancer cells.

Paper II

Mouse development is not obviously affected by the absence of dermatan sulfate epimerase 2 in spite of a modified brain dermatan sulfate composition

CS/DS has been implicated in neuronal plasticity where chondroitinase ABC treatment favors neuronal regeneration (182). After central nervous system injury, the levels of CS/DS increases. In situ hybridization of the developing brain suggested that DS-epi2 is the main DS-epimerase expressed during brain development (164), and interestingly, IdoA has been shown to promote neurite outgrowth (183). Furthermore, point mutations in DS-epi2 have been implicated in the bipolar disease type II (184). In this paper, we generated DS-epi2-deficient mice, and hypothesized that these mice would have an altered brain morphology and/or neuronal dysfunction.

Results

Dsel (*Dse-like*) gene, coding for DS-epi2, was disrupted by insertion of a targeting vector containing a neomycin-resistance cassette. Subsequently, ES clones were screened to confirm homologous recombination.

DS-epi2-deficient mice were found to be viable and were born with the expected Mendelian frequency. No difference in mortality was observed and the major organs displayed no histological abnormalities. DS epimerase activity was reduced in all tissues tested. Brain had the greatest reduction (89%) in DS-epimerase activity, followed by kidney (45% reduction). In this study, we confirmed earlier results that that DS-epi2 is the main DS-epimerase active in the brain.

To determine the amount of IdoA in tissues, ^{35}S-sulfate was injected into three-day-old mice. The labeled CS/DS chains were recovered and cleaved to establish the amount and distribution of IdoA residues. Brain contained low amounts of IdoA (2%). The IdoA amount decreased by 38% in DS-epi2 -/- brains.

DS-epi2 has a C-terminal sulfotransferase domain. Our group established that DS-epi2 does indeed have sulfotransferase activity

(unpublished results), but the position of the added sulfate has not yet been investigated. Interestingly, CS/DS in DS-epi2-/- brain had slightly altered sulfation patterns. DS-epi2-/- brain presented a reduction of 2% in mono-4-O-sulfated disaccharides and 2% upregulation of mono-6-O-sulfated disaccharides. Moreover, brain tissue was examined histologically and immunohistologically but no histological abnormalities were detected. Distribution of CS/DS was evaluated with CS56 (anti-CS) and Wisteria floribunda agglutinin lectin (WFA, recognizing N-acetyl-galactosamine), and no differences were found relative to control brain. Perineuronal nets were visualized by anti-aggrecan antibody in both control brain and DS-epi2-/- brain, and no differences were found.

In conclusion, we found that DS-epi2 is the main epimerase in the brain. Surprisingly, the IdoA content was only affected to a moderate extent when comparing DS-epi2-/- to WT brain. Furthermore, no alterations in brain structure were detected.

Paper III

The role of dermatan sulfate in migration of aortic smooth muscle cells

Based on the results from paper I, we next set out to elucidate the influence of IdoA and CS/DS-PGs on cellular properties, especially regarding migration. Aortic smooth muscle cells (AoSMCs) derived from DS-epi1-/- and WT mice were used in this study. These cells have been thoroughly studied, especially in the context of atherosclerotic plaques. In particular, CS/DS secreted by AoSMCs has been shown to bind and retain oxidized-LDL, which in turn is a primary cause of the development of plaques. Here we wanted to study the localization of CS/DS-PGs involved in migration and their mechanisms of action.

Results

DS epimerase activity was reduced to one fifth in DS-epi1 -/- AoSMCs compared to WT cells. Consequently, IdoA content was reduced in secreted CS/DS-PGs and in cell-surface CS/DS. DS-epi1-deficient cells migrated less than WT cells and to establish whether secreted or cell surface bound PGs influence migration, a number of experiments were performed. To study the role of secreted CS/DS-PGs, WT and DS-epi1-/- AoSMCs were allowed to migrate in the presence of conditioned medium derived from DS-epi1 -/- and WT cells. The conditioned medium had no influence on migration of the cells of either genotype. Both cell types were seeded on DS-epi1 -/- and WT matrices to examine the role of matrix-derived CS/DS-PGs. Interestingly, the different matrices did not

effect migration. As neither secreted or matrix-derived IdoA affected migration, we concluded that cell-surface-bound IdoA-containing PGs influence migration. The actin cytoskeleton and focal adhesions are essential structures in migration. Migrating DS-epi1 -/- cells had disrupted actin stress fiber organization and seemed to partially have lost directionality, as they displayed protrusions in all directions.

In summary, we found that IdoA in cell-surface bound CS/DS-PGs participates in cell migration. Further studies are necessary to determine which PGs are involved in this function.

Ongoing studies

Paper I revealed a general increase of CS/DS in ESCC biopsies compared to normal tissue, as well as an insight into biological functions of IdoA produced by cancer cells. CS/DS-PGs are also present in the ECM produced by stromal cells. As previously described, the composition of the cancer stroma greatly influence tumor growth. Therefore, we are in the process of investigating the effect of stromal IdoA in tumor growth and angiogenesis.

Preliminary results

Syngeneic Lewis lung carcinoma cells were inoculated into the flank of DS-epi1 -/-, HET and WT mice, in a mixed C57BL6/129Sv genetic background. Preliminary data shows that tumors from DS-epi1-deficient mice weigh twice as much than tumors from WT mice (figure 5).

As previously mentioned, angiogenesis is necessary for tumor growth and wound healing. Many growth factors are known to promote vessel sprouting, but FGF2 specifically binds to IdoA containing CS/DS structures. CS/DS is abundantly released into human wound fluid and supports FGF2-mediated cell proliferation (185). Interestingly, when cultivating pieces of thoracic aortas from WT and DS-epi1-/- mice in the presence of FGF2, we found increased *ex vivo* microvessel sprouting in DS-epi1-/- aortas compared with WT aortas (figure 6).

The IdoA content was previously quantified in the entire ESCC biopsy (Paper I). However, the IdoA-containing compartments within the biopsies were not established. A phage display anti-IdoA antibody reacting towards CS/DS structures containing large amounts of IdoA was utilized in immunofluorescent staining (186). In the tumor tissue, the epitope was predominantly present in the stromal compartment and most likely associated with collagen fibrils (figure 7).

Conclusively, these preliminary experiments indicate that IdoA-blocks are especially present in the cancer stroma and reduction of IdoA structures may result in an increased syngeneic tumor growth and *ex vivo* angiogenesis. Further studies aim to elucidate the mechanisms of these functional observations.

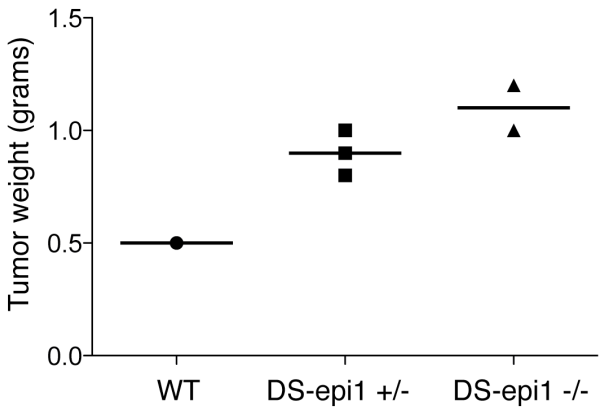


Figure 5. Increased syngeneic tumor growth in DS-epi1-/- mice. 1x10^6 GFP-Lewis lung carcinoma cells were subcutaneously injected into the right flank of WT, DS-epi1+/- and DS-epi1-/- mice. Animals were sacrificed after 18 days and the tumor mass was determined.

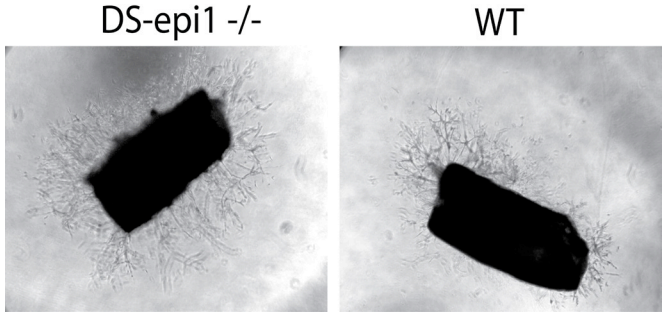


Figure 6. Increased *ex vivo* angiogenesis in DS-epi1-/- aortic rings. Thoracic mouse aortas from DS-epi1 -/- and WT mice were dissected and cleaned from the surrounding fibroadipose tissue. Aorta pieces (n = 6 from each genotype) were embedded in growth factor-reduced Matrigel and overlaid with FGF2-containing MCDB medium for 6 days (original magnification X 25).

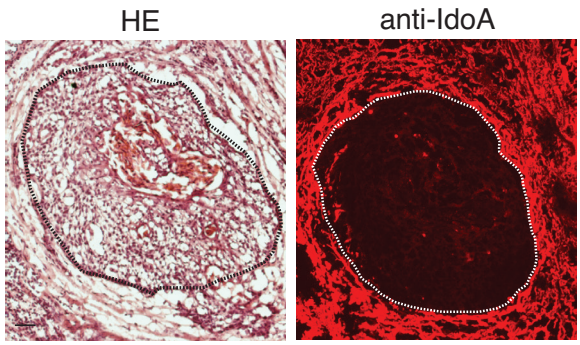


Figure 7. IdoA-containing epitope recognized by GD3A12 localizes to the stromal compartment. ESCC tumor tissue biopsies were cryo-sectioned and stained with hematoxylin and anti-IdoA antibody. Dotted line indicates the boundary between cancer cells and the surrounding stromal tissue. Cancer cells are encircled.

General discussion

This thesis have investigated the role of IdoA in biological processes has been investigated. Below follows a condensed summary of the major findings and a discussion of their biological and potential therapeutic implications.

DS-epimerase 1 in cancer development

Previous studies have defined DS-epi1 as a cancer specific antigen. We found that DS-epimerase activity and DS-epi1 expression was increased in cancer tissues compared to normal tissues (Paper I). In examined bioptic tissues, a number of different cell types, such as fibroblasts, cancer cells and immune cells, could contribute to the increased epimerase activity. The immunohistochemistry analysis of DS-epi1 showed expression both on cancer cells and in the stroma. Interestingly, the expression of DS-epi1 in the ESCC cell line was approx. 10 times greater compared to fibroblasts (HFL-1) (unpublished data). HFL-1 and macrophages are able to produce CS/DS-PGs with an IdoA content of 50% (179) (187) whereas cancer and embryological epithelial cells produce chains with approx. 15% (Paper I) and 5% (178), respectively. In our study, although DS-epimerase activity in tumor tissues was upregulated, the IdoA content remained the same in cancer compared to normal tissue. Hence, in epithelial cancer cells and cancer tissues, DS-epi1 abundance is not directly reflected in IdoA content. One possible explanation may be that cancer cells may not produce sufficient amount of D4ST-1 required for high IdoA production, preventing back epimerization (4). Cancer and normal tissues were analyzed for 4-*O*-sulfotransferase activity using either chondroitin or dermatan as substrates. Dermatan is also a substrate for D4ST-1, in addition to other STs, whereas chondroitin is not. Indeed, cancer tissues showed a 13-fold upregulation using chondroitin and a 3-fold upregulation using dermatan, indicating a shortage of D4ST-1. Another explanation for the discrepancy could be that isolated IdoA structures were not included in the mass spectrometry analysis, which analyzed only the IdoA-block and alternating structures (figure 2). However, other studies have obtained similar ratios of GlcA and IdoA in cancer versus normal tissues using other methods, arguing against this plausible explanation (112).

We investigated the role of stromal derived CS/DS with high density of IdoA in tumor tissues. Immunofluorescence analysis showed

that IdoA is predominately found along collagen fibrils, while significantly less IdoA epitope is associated with the tumor cells (Figure 7). As the antibody recognizes IdoA-blocks containing CS/DS structures (186), the presence of alternating IdoA/GlcA and isolated IdoA structures could not be determined. Interestingly, preliminary data indicate that DS-epi1-deficient mice had increased tumor growth compared to WT. The differences in tumor growth can potentially be explained by growth factor sequestering by IdoA. Reduced IdoA might result in increased growth factor availability on cancer cells. These growth factors could then stimulate cancer cells to proliferate, invade and migrate. HGF and FGF2 are two cancer-promoting growth factors known to interact with IdoA. Interestingly, only HGF promoted migration in ESCC cells whereas FGF2 did not (Paper I). One explanation could be that cancer cells lack IdoA-blocks required for FGF2 binding (188). HGF, on the other hand, only needs a single IdoA residue (120). Macrophages are described to secrete PGs with a significant portion of IdoA (187). These PGs could participate in growth factor mediated migration gradients in analogy with the previously described EGF gradient (90).

Tumor growth is largely dependent on angiogenesis and FGF2 is a known mediator of angiogenesis. During wound healing CS/DS-containing IdoA is secreted and cooperates with FGF2. We found that IdoA-reduced aortas displayed increased angiogenesis in the presence of FGF2 compared to WT mice (Figure 6). In a recent study, CS/DS was able to rescue HS-deficiency (EXT1-/- mice) in embryoid aortic sprouting assays (41). Upon HS-deficiency, the CS/DS production was upregulated 2-fold and decorin mRNA expression was upregulated 5-fold. This compensation indicates that decorin may have regulatory functions in angiogenesis. Decorin is a secreted PG, which contains large amount of IdoA-blocks. Potentially, the increased *ex vivo* angiogenesis in DS-epi1-/- aortas could be a result of reduced sequestering of FGF2 by decorin compared to WT aortas.

A less investigated aspect is the regulation of DS-epimerases. However, a number of growth factors (i.e. TGF-beta, EGF, and, PDGF) are known to regulate epimerase activity in fibroblasts (9) and are abundantly expressed in cancers. Interestingly, in endothelial cells, DS-epi1 is upregulated under hypoxic conditions (unpublished results). This might increase the IdoA-content in endocan, which is mainly produced by endothelial cells.

Therapeutic implications in cancer

Cancer is primarily managed by surgery, chemotherapy and radiation. New therapeutic approaches are continuously emerging such as peptide-based immunotherapy. The concept is to activate immune cells by subcutaneous injections of tumor specific peptides. The administrated peptides are presented by antigen presenting cells, which activate cytotoxic T lymphocytes (CTLs). These peptides are presented on the cell surface, bound to the major histocompatibility complex (MHC) also named human leukocyte antigen (HLA) in humans. CTLs eliminate cancer cells that express these peptides on their A-, B-, or C-HLA receptors. These peptides often derive from proteins that are either overexpressed or aberrantly expressed in cancer. DS-epi1 was first cloned as a tumor specific antigen expressed on HLA-A24 (i.e. a subclass of the MHC class I receptors). Peptides derived from DS-epi1 have been used in clinical trials in glioblastoma multiforme (189) and prostate cancer (190), in combination with peptides derived from other cancer antigens. Thus, the clinical outcome might not only be attributed to DS-epi1 peptides. However, recently a peptide vaccine study on hepatocellular carcinoma exclusively used DS-epi1 peptides. This study showed a tendency of lower recurrence rate in patients with increased CTL activities towards DS-epi1 peptides.

Cancers frequently have mutated or truncated proteins. Two fusion forms of C4ST-1, a CS/DS biosynthetic enzyme, were present in B-cell chronic lymphocytic leukemia (191). It was speculated that these truncated proteins were secreted, as they lacked certain domains. Consequently, DS-epi1 might be truncated or mutated in cancer cells, resulting in increased peptide presentation. Hypothetically, mutated forms of DS-epi1 might be secreted from tumor cells. Since DS-epi1 acts on non-sulfated chondroitin substrates in the Golgi, an extracellular activity of DS-epi1 is unlikely. Moreover, as DS-epi1 is highly expressed in most cancers investigated, DS-epi1 might be a prognostic marker. Therefore, it would be interesting to determine if DS-epi1 is present in blood from cancer patients.

Another potential therapeutic approach could be modulation of the HGF/MET axis by IdoA reduction. Currently, no specific inhibitors or antibodies directed against the HGF/MET axis are available in the clinic. However, a great number of clinical trials are ongoing and a few have reached phase III, for example treatment of non-small lung cancer and medullary thyroid cancer (192). Interestingly, a clinical trial of esophagus adenocarcinoma using anti-HGF antibody in combination with chemotherapy has reached phase II. Development of a DS-epi1 inhibitor to downregulate the HGF/MET axis could potentially be used in combination with chemotherapy. An ongoing project in the lab is the

search for DS-epi1 inhibitors, which could potentially inhibit the HGF/MET axis.

IdoA biosynthesis

In humans, DS-epi1 and DS-epi2 are ubiquitously expressed, although with preferences for different tissues. As described previously, IdoA can be displayed in different patterns i.e. IdoA-blocks, alternating GlcA/IdoA structures or isolated IdoA residues (figure 2). One of the main focuses in our lab is to determine the roles of the two DS-epimerases in IdoA formation. On a biochemical level DS-epi1 and DS-epi2 have slightly different features depending on the cell type studied. Fibroblasts produce substantial amounts of IdoA-blocks. Silencing of either DS-epi1, DS-epi2 or D4ST-1 in fibroblasts results in reduced IdoA-blocks and increased alternating structures (179). The alternating GlcA/IdoA residue formation is suggested to only require DS-epi1 or DS-epi2. Epithelial cells produce less IdoA and overexpression of DS-epi1, but not DS-epi2, results in increased IdoA-block formation.

The distinct functions of DS-epi1 and DS-epi2 *in vivo* are dependent on the investigated tissue. Ablation of DS-epi1 results in loss of IdoA-blocks when evaluating the IdoA content in the entire mouse (6). Moreover, DS-epi1-/- skin decorin is almost devoid of IdoA-blocks whereas the alternating IdoA structures are increased. Furthermore, DS-epi2-/- brain is reduced in alternating structures, whereas DS-epi2-/- kidney has a slight decrease of IdoA-block structures (Paper II). Conclusively, *in vivo,* two epimerases can catalyze all types of IdoA structures independently of each other, although DS-epi1 is essential to generate large amounts of IdoA-blocks. DS-epi2, on the other hand, is primarily responsible for alternating GlcA/IdoA structures *in vivo*.

Adding another level of complexity, IdoA content depends on the core protein. Potentially, CS/DS-PGs have inherited core protein information, resulting in different degrees of epimerization. A couple of different mechanistic explanations are proposed; one theory suggests that the biosynthetic enzymes are recruited to the core protein whereas the other theory proposes different sub-compartments of enzymes in the Golgi to which the core protein is guided. Xylosides, chemical compounds which lack a protein core, are primed but more importantly the primed GAG is epimerized to a low degree resembling the IdoA content found on versican (unpublished results) (193). Moreover, DS-epi1 and D4ST-1 are shown to co-localize in a certain Golgi compartment i.e. cis-Golgi (unpublished results). These results might indicate that the CS/DS bioenzymatic machinery in the Golgi is composed of sub-compartments of enzymes, although further efforts are needed to conclusively determine how CS/DS is assembled.

Biological functions of IdoA in the brain

CS/DS is a major component of brain matrix and has been described both to inhibit and favor regenerative processes. For instance, CS/DS is believed to be a barrier for axon penetration upon spinal injury (182). Another function of CS/DS is axon guidance, as it establishes permissive sprouting paths. Disulfated residue-containing domains have been shown to be essential for neuronal sprouting (194). The CS/DS-PGs are reported to contain low amounts of IdoA; 1-2% in the adult brain, whereas the embryonic brain contains 8-9% IdoA. Domains containing both IdoA and disulfated residues were found in brain embryos. These embryonic CS/DS structures possess high neurite sprouting activity compared to adult brain CS/DS. DS-epi2-/- brain, despite an approx. 40% decrease of IdoA, is histologically normal. The ECM was examined by immunofluorescent staining of CS, GalNAc, and aggrecan and no differences were found compared to WT brain (Paper II). Our results indicate that brain development possesses compensatory mechanisms. A dramatic compensation was also found in the quadruple knock-out of neurocan, brevican, tenascin-R and tenascin-C, as these mice presented very mild phenotypes (195). Nevertheless, IdoA might be important for neonatal brain development as indicated by its higher expression in brain embryos compared to adult brain. Neonatal brain is very plastic. Consequently, challenge of brain lacking DS-epi2 in animal models of brain lesions might lead to reduced abilities of neural regeneration in DS-epi2-/- compared to WT brain.

The perineuronal nets (PNNs) are specialized ECM substructures surrounding certain neuronal cell bodies and proximal dendrites. These dense networks consist of CS/DS-PGs (e.g. brevican, neurocan and aggrecan), hyaluronan, tenascin-R and link proteins (196). Different functions of CS/DS-PGs in PNNs are suggested such as sequestering of plasticity-promoting growth factors, presentation of inhibitory molecules like semaphorins and restriction of membrane protein mobility such as AMPA receptors. PNNs were detected in the expected locations in DS-epi2-/- brain and to the same extent compared to WT brain.

Interestingly, DS-epi2 is genetically associated to human type II bipolar disorder (184). Hence, provocation of DS-epi2-/- mice in models of mania and depression, such as sleep deprivation or forced swim test, might guide us in new directions. Autism, another example of a complex behavioral disease, is described after conditional deletion of the HS-polymerase, EXT1, in brain (197). The growth factor brain-derived neurotrophic factor (BNPF) plays a key role in mood regulation (198) and is decreased in the hippocampus of animals displaying bipolar disorder-related behavioral abnormalities such as depression and anxiety (199).

Hippocampus is the only brain structure retaining the disulfated structure iD units in adulthood (165). As DS-epi2 comprises a sulfotransferase domain, it could be postulated that DS-epi2 generates specific disulfated structures such as the iB, iD or iE units, modulating neurotropic factors implicated in mood disorders. Nevertheless, structure analysis of total DS-epi2-/- brain shows no substantial differences in disulfated residues compared to WT brain. However, no conclusion whether DS-epi2 generates these structures *in vivo* could be drawn from the disaccharide analysis, as other sulfotransferases could compensate for the loss of DS-epi2. To establish the exact position of the added sulfate by DS-epi2, isolated recombinant enzyme will be required.

IdoA in migration and adhesion

As previously mentioned, glycosaminoglycan chains regulate and bind growth factors as well as collagens. In addition, transmembrane PGs are associated with migration and adhesion. Their cytoplasmic domains are binding sites for cytoskeletal proteins and kinases. A number of transmembrane CS/DS-PGs (e.g. CSPG4, CD44, syndecans, and neuropilin-1) are known to facilitate migration . The presence of IdoA is overlooked in most of these CS/DS-PGs (Table I), although CD44 and syndecan-1 are known to carry IdoA. CD44 is a part-time PG, occurring as a protein lacking the GAG chains and in a proteoglycan form, carrying both HS and CS/DS chains. Upon PDGF stimulation, CD44 is reported to express IdoA, which might mediate migration of fibroblasts (10). Moreover, CD44 is known to interact with both the MET receptor and the cytoskeleton (58). A CD44 isoform, CD44v6, is shown to attract the ERM proteins (i.e. erzin, radixin, moesin) to the cytoplasmic tail, which are essential for signaling transduction of the MET receptor (159). Upon HGF-dependent activation, the ERM proteins bind to the cytoskeleton and mediate RAS activation by the guanine nucleotide exchange factor SOS (figure 8). The HS-carrying CD44 isoform, CD44v3, is proposed to facilitate FGF-4 and FGF-8 signaling by the HS chain (200). The role of HS in MET-signaling is contradictory, as one study showed that it participates in HGF signaling whereas another study showed the opposite effect (158,159). None of these reports considered a regulatory function of the CS/DS chain. A working hypothesis, resulting from the data reported in this thesis, is that the IdoA-containing CS/DS chain on CD44 binds HGF and presents it to the MET receptor, resulting in activation of the signaling pathway, followed by re-organization of the cytoskeleton and adhesion complexes (figure 8). We demonstrated that pERK1/2 and its downstream target RSK1/2/3 were induced after HGF stimulation (Paper I). DS-epi1 downregulated cells had greater pERK1/2 expression than control cells. Moreover, DS-epi1-silenced cells had reduced

migration and disrupted cytoskeleton architecture in comparison with control cells. This might be mediated by RSK1/2/3 and the downstream target filamin A, which is known to determine cell shape and locomotion. In addition, CD44 is localized at the leading edge and in lamellipodia (201).

DS-epi1-silenced cells had increased cell areas compared to control cells (Paper I). Upon DS-epi1 silencing, the ESCC cell line upregulated the mRNA expression of the β3 integrin unit 5-fold compared to control cells (unpublished results). Moreover, DS-epi1-downregulated cells had approx. 2-fold induction of FAK and pFAK expression compared with control cells. Several potential IdoA-containing PGs could be involved in adhesion. Another explanation could be a regulatory function of DS-epi1 that upregulates for example β3 integrin unit, resulting in increased cell spreading.

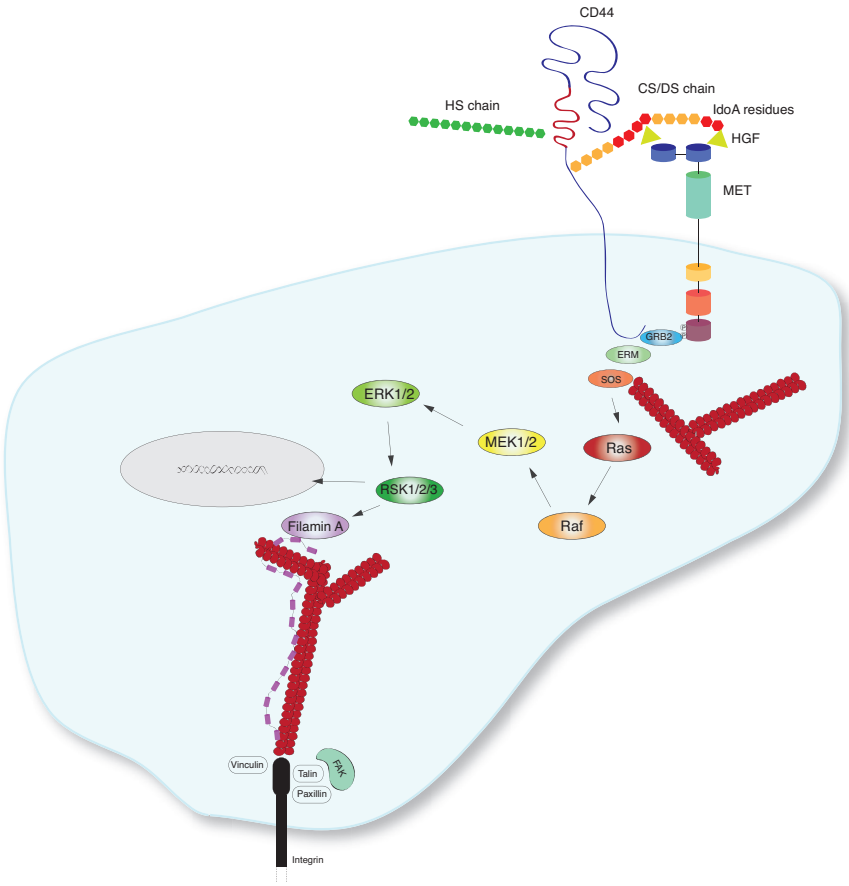


Figure 8. Working hypothesis — IdoA in CS/DS mediated HGF-signaling. CD44-associated IdoA binds and presents HGF to the MET receptor, which elicits downstream signaling. This results in re-organization of actin cytoskeleton and focal adhesion complexes.

Future perspectives

The chondroitin/dermatan sulfate field has developed considerably during the last decades. After identification and cloning of the two DS-epimerases, the role IdoA could be investigated in loss-of-function models. Studies are ongoing in our lab to further elucidate the role of DS-epi1 in inflammation and embryogenesis. Recently, we generated a DS-epi1 and DS-epi2 double knock-out, supposedly totally devoid of IdoA. Surprisingly, these mice are vital, although smaller in size, being reduced approx. 40% in size and weight. This animal model could be used to study the role of IdoA in processes like angiogenesis, coagulation, wound healing, atherosclerosis, embryogenesis and tumorigenesis. Moreover, *in vivo* produced CS/DS structures depleted of IdoA could be used in growth factor binding and interaction studies. One setup could be to attach CS/DS with and without IdoA to a column and apply conditioned media from cells. Mass spectrometry would then be run on the bound fractions to determine novel binding molecules for IdoA.

Another approach to study IdoA is to generate of DS-epi1 and/or DS-epi2 inhibitors to block the formation of IdoA. An ongoing project in the lab is to crystallize DS-epi1, which will then be used to model potential inhibitors. These inhibitors could be studied in tumorigenic models. Would they promote or inhibit tumor formation? Is migration and invasion affected *in vivo* by an DS-epi1 inhibitor? Which CS/DS-PG or CS/DS-PGs mediate cell migration?

Another interesting aspect of DS-epi1 in tumor development is the fact that peptides derived from DS-epi1 has been used in clinical trials. Why do these tumor associated peptides elicit CTL responses? Could DS-epi1 possess another function in tumors beside IdoA formation? Since DS-epi1 is overexpressed in cancers, it would be exciting to see if it is detectable in the blood of cancer patients and be used as diagnostic/prognostic markers. In a therapeutic perspective, disturbing the interaction of IdoA with HGF could be of great potential. Targeting the MET/HGF axis has attracted much attention and modulation of IdoA could be one approach to this aim. Therefore, DS-epi1 inhibitors could be of great interest.

Since IdoA is increased during fetal brain development, it is plausible that it mediates important functions. Consequently, the IdoA deficient double knock-out mice might reveal altered brain structure or even neuropsychiatric disorders. Human psychiatric diseases cannot be

sufficiently replicated in mouse models, although they are still valuable tools. Provoking DS-epi2 -/- or the double knock-out, for example in sleep deprivation models, could guide us in novel directions. Conclusively, the role of IdoA in biological processes is a growing field, and could lead to novel therapeutics.

Populärvetenskaplig sammanfattning

Under sin livstid kommer var tredje svensk i personlig kontakt med någon tumörform. För att effektivare kunna behandla cancer behöver vi bättre förstå de bakomliggande mekanismerna. Varför växer en cancer och hur sker spridningen till andra organ? En cancervävnad består av ett stort antal olika celler som ständigt kommunicerar med varandra. I en frisk vävnad finns reglerande mekanismer som hindrar att celler växer okontrollerbart. Dessa mekanismer saknas i en cancervävnad. Vi har studerat matstrupscancer, som är den sjunde vanligaste cancer relaterade dödsorsaken i världen. Denna tumörform har dålig prognos och generellt överlever patienterna endast en kortare tid efter att de fått diagnosen.

Vanligtvis när cancerutveckling studeras så intresserar man sig för proteinernas funktioner. Vi har istället koncentrerat oss på kolhydratstrukturer som är förankrade på proteiner. Proteinerna sitter dels fast på cellernas yta samt finns även i vävnaden runt omkring celler i det så kallade extracellulära matrixet. Uppbyggnaden av de långa kolhydratkedjorna sker inuti cellen och under processen modifieras dessa kedjor av olika enzymer. Vi har studerat två av dessa enzymer som utför en typ av modifiering. De katalyserar en konformationsändring hos kolhydraterna varefter proteinerna transporteras ut ur cellen eller upp till cellytan.

Denna avhandling behandlar hur dessa två enzymer påverkar olika biologiska processer. Enzymerna heter DS-epimerase 1 och 2. De utför en modifikation på kolhydratstrukturen kondroitinsulfat och generar istället dermatansulfat. Framförallt behandlas funktionen av DS-epimerase 1 med avseende på utveckling av tumörer. Enzymet är fyra gånger så aktivt i vävnad från matstrupscancer jämfört med frisk vävnad. Dermatansulfat har en viktig betydelse inom tumörsignalering. Dermatansulfat binder en speciell signalmolekyl och presenterar troligen denna molekyl för cellen, varefter signalerna påverkar cancercellens rörelse. När DS-epimerase 1 blockeras så hindras bildningen av dermatansulfat och detta leder till att cancercellen rör sig mindre, vilket kan påverka cancercellens metastasering. I laboratoriet pågår experiment att utveckla en hämmare mot DS-epimerase 1, därigenom skulle möjligtvis cancercellernas rörelse minska samt deras spridning i kroppen. Det krävs dock mycket forskning innan ett sådant läkemedel skulle kunna användas kliniskt.

Den andra delen av avhandlingen handlar främst om hur hjärnans utveckling påverkas av dermatansulfat. DS-epimerase 2 finns framförallt i hjärnan och tidigare studier har visat att mutationer i genen för DS-

epimerase 2 finns överrepresenterad hos patienter som lider av en viss typ av manodepressiv sjukdom. För att studera DS-epimerase 2 normala biologiska funktioner genmodifierade vi en mus så att den saknar detta enzym. När vi undersökte mushjärnan fann vi inga uppenbara skillnader i jämförelse med en normal mushjärna. Nya experiment krävs för att utröna dess biologiska funktioner. Framförallt skulle det vara intressant att använda denna mus i experiment där man framkallar symptom som manodepressiva patienter lider av. Först då kan vi se ifall enzymet har en bidragande funktion till manodepressiv sjukdom.

Sammanfattningsvis visar min avhandling på möjligheter att angripa cancerutveckling ur ett nytt perspektiv. Ett läkemedel som blockerar DS-epimerase 1 skulle möjligtvis kunna användas i kombination med andra läkemedel för att förbättra överlevnaden hos cancerpatienter.

Acknowledgements

I would like to express my thanks to all those who have helped me during my work with this thesis. All members of BMC and especially those at the old C13 floor and the present D12 floor. In particular I would like to thank:

Anders Malmström, my supervisor, for always supporting and believing in me. You truly see possibilities in all situations and your optimism can really move mountains. Thanks for always keeping your door open!

Marco Maccarana, my co-supervisor, for your vast scientific knowledge and for your guidance in the lab; everything from experimental setups to the importance of staying well-organized.

Mef Nilbert, my-co supervisor, for support and encouragement.

Mattias Belting, for generously sharing your scientific expertise during a critical resubmission period.

Johan Malmström, for the mass spectrometry collaboration and for stimulating discussions.

Gunilla Westergren-Thorsson, for your support over the years.

Past and present members of Malmström and Westergren-Thorsson lab for providing a stimulating working environment and laughs. *Benny,* my older research brother, who during my first years as PhD student really helped and guided a confused medical student through experimental design setups and always took time to answer my questions. *Erik*, my research ”uncle”, for supporting chats and guidance. And you really know how to get a party going! *Jakob* for bringing new positive energy into the lab and providing a helping hand during a critical time. *Emil* for introducing me to the field of chemistry and for making the best pretzel. *Barbara, Giacomo, and Mariam* for collaborations and friendship. *Sara, Jenny, Oskar, Annika, Marie, Lena, Kristina, Anna-Karin, Maria, Anna, Kristian, Ellen, Camilla,* and *Olivia* for chats and laughter over the years.

I would also like to acknowledge our international collaborators. In particular *Joe Zaia and Xiaofeng Shi* for work involving quantification of CS/DS in Paper I.

Outside the lab I really would like to express my greatest gratitude to:

My friends - you all keep a special place in my heart. *Christian* and *Olof* for great times in Prague and all other places. *Henrik* for great tennis matches and *Josef* for a crazy time in Venezuela. And all my other friends from medical school and their partners for great laughters, parties and friendships. My high school friends *Andreas and Jakob,* we need to hang out more often. Better friends cannot be found!

My family; Mum and Dad for love, support and guiding me to the person I am today. *Johanna and Gustaf* for being loving and kind. You are the best sister and brother. *Anders, Karin, Lea, Elin, and Siri* for being just the persons you are and for bringing laughter and happiness. Also, *Erik and Jorun,* you belong to family. *Erik* for being like a brother to me and for all fun times. You are all fantastic!

The extra family provided by *Katrin,* thanks for great times *Satu, Mats, André, Rune and Anita,* for being warm, kind and letting me be the person I am.

And the most important person of all; *Katrin*, thanks for always supporting me. I dedicate this thesis to you as I truly love you and for your way of keeping me on the right track. You make me a better person; I love you!

References

1. Prabhakar V, Sasisekharan R. The biosynthesis and catabolism of galactosaminoglycans. Adv. Pharmacol. 2006;53:69–115.

2. Mizuguchi S, Uyama T, Kitagawa H, Nomura KH, Dejima K, Gengyo-Ando K, et al. Chondroitin proteoglycans are involved in cell division of Caenorhabditis elegans. nature. 2003May22;423(6938):443–8.

3. Pacheco B, Maccarana M, Goodlett DR, Malmström A, Malmstrom L. Identification of the Active Site of DS-epimerase 1 and Requirement of N-Glycosylation for Enzyme Function. Journal of Biological Chemistry. 2008Nov.20;284(3):1741–7.

4. Malmström A. Biosynthesis of dermatan sulfate. II. Substrate specificity of the C-5 uronosyl epimerase. J. Biol. Chem. 1984Jan.10;259(1):161–5.

5. Pacheco B, Maccarana M, Malmström A. Dermatan 4-O-sulfotransferase 1 is pivotal in the formation of iduronic acid blocks in dermatan sulfate. Glycobiology. 2009Oct.5;19(11):1197–203.

6. Maccarana M, Kalamajski S, Kongsgaard M, Magnusson SP, Oldberg A, Malmström A. Dermatan Sulfate Epimerase 1-Deficient Mice Have Reduced Content and Changed Distribution of Iduronic Acids in Dermatan Sulfate and an Altered Collagen Structure in Skin. Molecular and Cellular Biology. 2009Sep.28;29(20):5517–28.

7. Zhang L, Müller T, Baenziger JU, Janecke AR. Congenital disorders of glycosylation with emphasis on loss of dermatan-4-sulfotransferase. Prog Mol Biol Transl Sci. 2010;93:289–307.

8. Miyake N, Kosho T, Mizumoto S, Furuichi T, Hatamochi A, Nagashima Y, et al. Loss-of-function mutations of CHST14 in a new type of Ehlers-Danlos syndrome. Hum. Mutat. 2010Aug.;31(8):966–74.

9. Tiedemann K, Olander B, Eklund E, Todorova L, Bengtsson M, Maccarana M, et al. Regulation of the chondroitin/dermatan fine structure by transforming growth factor-beta1 through effects on polymer-modifying enzymes. Glycobiology. 2005Dec.;15(12):1277–85.

10. Clark RAF, Lin F, Greiling D, An J, Couchman JR. Fibroblast invasive migration into fibronectin/fibrin gels requires a previously uncharacterized dermatan sulfate-CD44 proteoglycan. J. Invest. Dermatol. 2004Feb.;122(2):266–77.

11. Seidler DG, Breuer E, Grande-Allen KJ, Hascall VC, Kresse H. Core protein dependence of epimerization of glucuronosyl residues in galactosaminoglycans. J. Biol. Chem. 2002Nov.1;277(44):42409–16.

12. Silbert JE, Sugumaran G. Biosynthesis of chondroitin/dermatan sulfate. IUBMB Life. 2002Oct.;54(4):177–86.

13. Mikami T. Specificities of Three Distinct Human Chondroitin/Dermatan N-Acetylgalactosamine 4-O-Sulfotransferases Demonstrated Using Partially Desulfated Dermatan Sulfate as an Acceptor: IMPLICATION OF DIFFERENTIAL ROLES IN DERMATAN SULFATE BIOSYNTHESIS. Journal of Biological Chemistry. 2003Jul.3;278(38):36115–27.

14. Kang H-G, Evers MR, Xia G, Baenziger JU, Schachner M. Molecular cloning and characterization of chondroitin-4-O-sulfotransferase-3. A novel member of the HNK-1 family of sulfotransferases. J. Biol. Chem. 2002Sep.20;277(38):34766–72.

15. Fukuta M, Kobayashi Y, Uchimura K, Kimata K, Habuchi O. Molecular cloning and expression of human chondroitin 6-sulfotransferase. Biochim. Biophys. Acta. 1998Jul.30;1399(1):57–61.

16. Tsutsumi K, Shimakawa H, Kitagawa H, Sugahara K. Functional expression and genomic structure of human chondroitin 6-sulfotransferase. FEBS Lett. 1998Dec.18;441(2):235–41.

17. Bhakta S, Bartes A, Bowman KG, Kao WM, Polsky I, Lee JK, et al. Sulfation of N-acetylglucosamine by chondroitin 6-sulfotransferase 2 (GST-5). J. Biol. Chem. 2000Dec.22;275(51):40226–34.

18. Uchimura K, Kadomatsu K, Nishimura H, Muramatsu H, Nakamura E, Kurosawa N, et al. Functional analysis of the chondroitin 6-sulfotransferase gene in relation to lymphocyte subpopulations, brain development, and oversulfated chondroitin sulfates. J. Biol. Chem. 2002Jan.11;277(2):1443–50.

19. Thiele H, Sakano M, Kitagawa H, Sugahara K, Rajab A, Höhne W, et al. Loss of chondroitin 6-O-sulfotransferase-1 function results in severe human chondrodysplasia with progressive spinal involvement. Proc. Natl. Acad. Sci. U.S.A. 2004Jul.6;101(27):10155–60.

20. Ohtake S, Ito Y, Fukuta M, Habuchi O. Human N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase cDNA is related to human B cell recombination activating gene-associated gene. J. Biol. Chem. 2001Nov. 23;276(47):43894–900.

21. Kobayashi M, Sugumaran G, Liu J, Shworak NW, Silbert JE, Rosenberg RD. Molecular cloning and characterization of a human uronyl 2-sulfotransferase that sulfates iduronyl and glucuronyl residues in dermatan/chondroitin sulfate. J. Biol. Chem. 1999Apr.9;274(15):10474–80.

22. Sugahara KN, Hirata T, Tanaka T, Ogino S, Takeda M, Terasawa H, et al. Chondroitin Sulfate E Fragments Enhance CD44 Cleavage and CD44-Dependent Motility in Tumor Cells. Cancer Research. 2008Sep.1;68(17): 7191–9.

23. Brown LF, Guidi AJ, Schnitt SJ, Van De Water L, Iruela-Arispe ML, Yeo TK, et al. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. Clin. Cancer Res. 1999May;5(5): 1041–56.

24. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011Mar.4;144(5):646–74.

25. Davies MA, Samuels Y. Analysis of the genome to personalize therapy for melanoma. Oncogene. 2010Oct.14;29(41):5545–55.

26. Junttila MR, Evan GI. p53--a Jack of all trades but master of none. Nat Rev Cancer. 2009Nov.;9(11):821–9.

27. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat. Rev. Genet. 2005Aug.;6(8):611–22.

28. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes & Development. 2009Mar.1;23(5):537–48.

29. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U.S.A. 2003Apr.1;100(7):3983–8.

30. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010Mar.19;140(6):883–99.

31. Zhou Y, Song B, Qin W-J, Zhang G, Zhang R, Luan Q, et al. Heparanase promotes bone destruction and invasiveness in prostate cancer. Cancer Letters. 2008Sep.18;268(2):252–9.

32. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature. 2001May17;411(6835):375–9.

33. Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. Proc. Natl. Acad. Sci. U.S.A. 2004Apr.6;101(14):4966–71.

34. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol. Rev. 2003Jul.;83(3):835–70.

35. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer. 2003Jun.;3(6):401–10.

36. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. J. Pathol. 2003Jul.;200(4):429–47.

37. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. Journal of Clinical Investigation. 1999Jan.;103(2):159–65.

38. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. The American Journal of Pathology. 2002Mar.;160(3):985–1000.

39. Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellén L, et al. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. Developmental Cell. 2006May;10(5):625–34.

40. Ashikari-Hada S, Habuchi H, Kariya Y, Kimata K. Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. J. Biol. Chem. 2005Sep.9;280(36):31508–15.

41. Le Jan S, Hayashi M, Kasza Z, Eriksson I, Bishop JR, Weibrecht I, et al. Functional Overlap Between Chondroitin and Heparan Sulfate Proteoglycans During VEGF-Induced Sprouting Angiogenesis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2012Feb.16.

42. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Research. 1989Dec.1;49(23):6449–65.

43. Tlsty TD. Stromal cells can contribute oncogenic signals. Seminars in Cancer Biology. 2001Apr.;11(2):97–104.

44. Heldin CH. Structural and functional studies on platelet-derived growth factor. EMBO J. 1992Dec.;11(12):4251–9.

45. Furuhashi M, Sjöblom T, Abramsson A, Ellingsen J, Micke P, Li H, et al. Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. Cancer Research. 2004Apr.15;64(8):2725–33.

46. Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M, Westermark B. Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc. Natl. Acad. Sci. U.S.A. 1993Jan.15;90(2):393–7.

47. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. J. Pathol. 2003Oct.;201(2):221–8.

48. Brinckerhoff CE, Matrisian LM. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol. 2002Mar.;3(3):207–14.

49. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002Mar.;2(3):161–74.

50. Borchers AH, Steinbauer H, Schafer BS, Kramer M, Bowden GT, Fusenig NE. Fibroblast-directed expression and localization of 92-kDa type IV collagenase along the tumor-stroma interface in an in vitro three-dimensional model of human squamous cell carcinoma. Mol. Carcinog. 1997Aug.;19(4):258–66.

51. Fuster MM, Esko JD. The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer. 2005Jul.;5(7):526–42.

52. Pierce DF, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ, et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc. Natl. Acad. Sci. U.S.A. 1995May9;92(10): 4254–8.

53. Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol. 2001Nov.;11(11):S44–51.

54. Tsushima H, Ito N, Tamura S, Matsuda Y, Inada M, Yabuuchi I, et al. Circulating transforming growth factor beta 1 as a predictor of liver metastasis after resection in colorectal cancer. Clin. Cancer Res. 2001May;7(5):1258–62.

55. Wesche J, Haglund K, Haugsten EM. Fibroblast growth factors and their receptors in cancer. Biochem. J. 2011Jul.15;437(2):199–213.

56. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocrine Related Cancer. 2004Dec.;11(4):709–24.

57. Memarzadeh S, Xin L, Mulholland DJ, Mansukhani A, Wu H, Teitell MA, et al. Enhanced paracrine FGF10 expression promotes formation of multifocal prostate adenocarcinoma and an increase in epithelial androgen receptor. Cancer Cell. 2007Dec.;12(6):572–85.

58. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol. 2010Dec.;11(12):834–48.

59. Tyan S-W, Kuo W-H, Huang C-K, Pan C-C, Shew J-Y, Chang K-J, et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PLoS ONE. 2011;6(1):e15313.

60. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell. 2003Apr.;3(4):347–61.

61. Hu YC, Lam KY, Law S, Wong J, Srivastava G. Profiling of differentially expressed cancer-related genes in esophageal squamous cell carcinoma (ESCC) using human cancer cDNA arrays: overexpression of oncogene MET correlates with tumor differentiation in ESCC. Clin. Cancer Res. 2001Nov.;7(11):3519–25.

62. Kammula US, Kuntz EJ, Francone TD, Zeng Z, Shia J, Landmann RG, et al. Molecular co-expression of the c-Met oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome. Cancer Letters. 2007Apr.18;248(2):219–28.

63. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science. 2003Dec.5;302(5651):1704–9.

64. Ridley AJ. Life at the leading edge. Cell. 2011Jun.24;145(7):1012–22.

65. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol. 2009Jul.;10(7):445–57.

66. Weijer CJ. Collective cell migration in development. Journal of Cell Science. 2009Sep.15;122(Pt 18):3215–23.

67. Welch MD, Mullins RD. Cellular control of actin nucleation. Annu. Rev. Cell Dev. Biol. 2002;18:247–88.

68. Gupton SL, Gertler FB. Filopodia: the fingers that do the walking. Sci. STKE. 2007Sep.8;2007(400):re5.

69. Buccione R, Caldieri G, Ayala I. Invadopodia: specialized tumor cell structures for the focal degradation of the extracellular matrix. Cancer Metastasis Rev. 2009Jun.;28(1-2):137–49.

70. Machacek M, Hodgson L, Welch C, Elliott H, Pertz O, Nalbant P, et al. Coordination of Rho GTPase activities during cell protrusion. Nature. 2009Sep.3;461(7260):99–103.

71. Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. Molecular and Cellular Biology. 2002Sep.;22(18):6582–91.

72. Rodriguez OC, Schaefer AW, Mandato CA, Forscher P, Bement WM, Waterman-Storer CM. Conserved microtubule-actin interactions in cell movement and morphogenesis. Nat Cell Biol. 2003Jul.;5(7):599–609.

73. Pertz O. Spatio-temporal Rho GTPase signaling - where are we now? Journal of Cell Science. 2010Jun.1;123(Pt 11):1841–50.

74. Heasman SJ, Carlin LM, Cox S, Ng T, Ridley AJ. Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. The Journal of Cell Biology. 2010Aug. 23;190(4):553–63.

75. Zaidel-Bar R, Geiger B. The switchable integrin adhesome. Journal of Cell Science. 2010May1;123(Pt 9):1385–8.

76. Legate KR, Montañez E, Kudlacek O, Fässler R. ILK, PINCH and parvin: the tIPP of integrin signalling. Nat Rev Mol Cell Biol. 2006Jan.;7(1):20–31.

77. Schlaepfer DD, Mitra SK, Ilic D. Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2004Jul.;1692(2-3):77–102.

78. Ilić D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. nature. 1995Oct.12;377(6549):539–44.

79. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer — a new therapeutic opportunity. Nat Rev Cancer. 2005Jul.;5(7):505–15.

80. Lu Z, Jiang G, Blume-Jensen P, Hunter T. Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. Molecular and Cellular Biology. 2001Jun.;21(12):4016–31.

81. Ilić D, Genbacev O, Jin F, Caceres E, Almeida EA, Bellingard-Dubouchaud V, et al. Plasma membrane-associated pY397FAK is a marker of cytotrophoblast invasion in vivo and in vitro. The American Journal of Pathology. 2001Jul.;159(1):93–108.

82. Hayashi K, Kadomatsu K, Muramatsu T. Requirement of chondroitin sulfate/ dermatan sulfate recognition in midkine-dependent migration of macrophages. Glycoconj J. 2001May;18(5):401–6.

83. Merle B, Durussel L, Delmas PD, Clézardin P. Decorin inhibits cell migration through a process requiring its glycosaminoglycan side chain. J. Cell. Biochem. 1999Dec.1;75(3):538–46.

84. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med. 2006Aug.;12(8):895–904.

85. Cameron MD, Schmidt EE, Kerkvliet N, Nadkarni KV, Morris VL, Groom AC, et al. Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Research. 2000May1;60(9):2541–6.

86. Tarin D, Price JE, Kettlewell MG, Souter RG, Vass AC, Crossley B. Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. Cancer Research. 1984Aug.;44(8):3584–92.

87. Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. nature. 1998Mar. 12;392(6672):190–3.

88. Wyckoff JB, Jones JG, Condeelis JS, Segall JE. A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. Cancer Research. 2000May1;60(9):2504–11.

89. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Research. 2005Jun.15;65(12):5278–83.

90. Wyckoff JB, Wang Y, Lin EY, Li J-F, Goswami S, Stanley ER, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Research. 2007Mar.15;67(6):2649–56.

91. Xue C, Wyckoff J, Liang F, Sidani M, Violini S, Tsai K-L, et al. Epidermal growth factor receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. Cancer Research. 2006Jan.1;66(1):192–7.

92. BORSIG L, Wong R, Feramisco J. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. 2001.

93. Nieswandt B, Hafner M, Echtenacher B, Männel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Research. 1999Mar.15;59(6):1295–300.

94. Karpatkin S, Pearlstein E, Ambrogio C, Coller BS. Role of adhesive proteins in platelet tumor interaction in vitro and metastasis formation in vivo. Journal of Clinical Investigation. 1988Apr.;81(4):1012–9.

95. Ibrahim T, Leong I, Sanchez-Sweatman O, Khokha R, Sodek J, Tenenbaum HC, et al. Expression of bone sialoprotein and osteopontin in breast cancer bone metastases. Clin Exp Metastasis. 2000;18(3):253–60.

96. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Research. 2002Mar.15;62(6):1832–7.

97. Morita M, Kumashiro R, Kubo N, Nakashima Y, Yoshida R, Yoshinaga K, et al. Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. Int. J. Clin. Oncol. 2010Apr.;15(2):126–34.

98. Polednak AP. Trends in survival for both histologic types of esophageal cancer in US surveillance, epidemiology and end results areas. Int. J. Cancer. 2003May20;105(1):98–100.

99. Lin SH, Chang JY. Esophageal cancer: diagnosis and management. Chin J Cancer. 2010Oct.;29(10):843–54.

100. Fukuzawa K, Noguchi Y, Yoshikawa T, Saito A, Doi C, Makino T, et al. High incidence of synchronous cancer of the oral cavity and the upper gastrointestinal tract. Cancer Letters. 1999Oct.1;144(2):145–51.

101. Chang-Claude J, Becher H, Blettner M, Qiu S, Yang G, Wahrendorf J. Familial aggregation of oesophageal cancer in a high incidence area in China. Int J Epidemiol. 1997Dec.;26(6):1159–65.

102. Burmeister BH, Smithers BM, Gebski V, Fitzgerald L, Simes RJ, Devitt P, et al. Surgery alone versus chemoradiotherapy followed by surgery for resectable cancer of the oesophagus: a randomised controlled phase III trial. Lancet Oncol. 2005Sep.;6(9):659–68.

103. Lin DC, Du XL, Wang MR. Protein alterations in ESCC and clinical implications: a review. Dis. Esophagus. 2009;22(1):9–20.

104. Xu X-C. Risk factors and gene expression in esophageal cancer. Methods Mol. Biol. 2009;471:335–60.

105. Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N, et al. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. Int. J. Cancer. 2006Mar.1;118(5):1173–80.

106. Takada N, Yano Y, Matsuda T, Otani S, Osugi H, Higashino M, et al. Expression of immunoreactive human hepatocyte growth factor in human esophageal squamous cell carcinomas. Cancer Letters. 1995Nov.6;97(2): 145–8.

107. Ren Y. Hepatocyte Growth Factor Promotes Cancer Cell Migration and Angiogenic Factors Expression: A Prognostic Marker of Human Esophageal Squamous Cell Carcinomas. Clinical Cancer Research. 2005Sep.1;11(17):6190–7.

108. Grugan KD, Miller CG, Yao Y, Michaylira CZ, Ohashi S, Klein-Szanto AJ, et al. Fibroblast-secreted hepatocyte growth factor plays a functional role in esophageal squamous cell carcinoma invasion. Proc. Natl. Acad. Sci. U.S.A. 2010Jun.15;107(24):11026–31.

109. Sarbia M, Stahl M, Fink U, Heep H, Dutkowski P, Willers R, et al. Prognostic significance of cyclin D1 in esophageal squamous cell carcinoma patients treated with surgery alone or combined therapy modalities. Int. J. Cancer. 1999Feb.19;84(1):86–91.

110. Mathew R, Arora S, Khanna R, Mathur M, Shukla NK, Ralhan R. Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer. European Journal of Cancer. 2002Apr.;38(6):832–41.

111. Zhao X-J, Li H, Chen H, Liu Y-X, Zhang L-H, Liu S-X, et al. Expression of e-cadherin and beta-catenin in human esophageal squamous cell carcinoma: relationships with prognosis. World J. Gastroenterol. 2003Feb.;9(2):225–32.

112. Theocharis AD, Vynios DH, Papageorgakopoulou N, Skandalis SS, Theocharis DA. Altered content composition and structure of glycosaminoglycans and proteoglycans in gastric carcinoma. The International Journal of Biochemistry & Cell Biology. 2003Mar.;35(3): 376–90.

113. Dam ten GB, van de Westerlo EMA, Purushothaman A, Stan RV, Bulten J, Sweep FCGJ, et al. Antibody GD3G7 selected against embryonic glycosaminoglycans defines chondroitin sulfate-E domains highly up-regulated in ovarian cancer and involved in vascular endothelial growth factor binding. The American Journal of Pathology. 2007Oct.;171(4): 1324–33.

114. Basappa, Murugan S, Sugahara KN, Lee CM, Dam ten GB, van Kuppevelt TH, et al. Involvement of chondroitin sulfate E in the liver tumor focal formation of murine osteosarcoma cells. Glycobiology. 2009Jul.;19(7): 735–42.

115. Li F, Dam ten GB, Murugan S, Yamada S, Hashiguchi T, Mizumoto S, et al. Involvement of highly sulfated chondroitin sulfate in the metastasis of the Lewis lung carcinoma cells. J. Biol. Chem. 2008Dec.5;283(49):34294–304.

116. Mizumoto S, Takahashi J, Sugahara K. Receptor for Advanced Glycation End Products (RAGE) Functions as a Receptor for Specific Sulfated Glycosaminoglycans, and Anti-RAGE Antibody or The Sulfated Glycosaminoglycans Delivered in vivo Inhibit Pulmonary Metastasis of Tumor Cells. Journal of Biological Chemistry. 2012Apr.9.

117. Monzavi-Karbassi B, Stanley JS, Hennings L, Jousheghany F, Artaud C, Shaaf S, et al. Chondroitin sulfate glycosaminoglycans as major P-selectin ligands on metastatic breast cancer cell lines. Int. J. Cancer. 2007;120(6): 1179–91.

118. KOZLOWSKI EO, PAVAO MSG, BORSIG L. Ascidian dermatan sulfates attenuate metastasis, inflammation and thrombosis by inhibition of P-selectin. J. Thromb. Haemost. 2011Sep.;9(9):1807–15.

119. Afratis N, Gialeli C, Nikitovic D, Tsegenidis T, Karousou E, Theocharis AD, et al. Glycosaminoglycans: key players in cancer cell biology and treatment. FEBS Journal. 2012Apr.;279(7):1177–97.

120. Deakin JA, Blaum BS, Gallagher JT, Uhrín D, Lyon M. The binding properties of minimal oligosaccharides reveal a common heparan sulfate/dermatan sulfate-binding site in hepatocyte growth factor/scatter factor that can accommodate a wide variety of sulfation patterns. J. Biol. Chem. 2009Mar.6;284(10):6311–21.

121. Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J. Biol. Chem. 2002Nov.15;277(46): 43707–16.

122. Fthenou E, Zafiropoulos A, Tsatsakis A, Stathopoulos A, Karamanos NK, Tzanakakis GN. Chondroitin sulfate A chains enhance platelet derived growth factor-mediated signalling in fibrosarcoma cells. The International Journal of Biochemistry & Cell Biology. 2006Jan.;38(12):2141–50.

123. Nikitovic D, Zafiropoulos A, Tzanakakis GN, Karamanos NK, Tsatsakis AM. Effects of glycosaminoglycans on cell proliferation of normal osteoblasts and human osteosarcoma cells depend on their type and fine chemical compositions. Anticancer Res. 2005Jul.;25(4):2851–6.

124. Denholm EM, Lin YQ, Silver PJ. Anti-tumor activities of chondroitinase AC and chondroitinase B: inhibition of angiogenesis, proliferation and invasion. Eur. J. Pharmacol. 2001Mar.30;416(3):213–21.

125. Iida J, Wilhelmson KL, Ng J, Lee P, Morrison C, Tam E, et al. Cell surface chondroitin sulfate glycosaminoglycan in melanoma: role in the activation of pro-MMP-2 (pro-gelatinase A). Biochem. J. 2007May1;403(3):553.

126. Pietras K, Östman A. Hallmarks of cancer: interactions with the tumor stroma. Experimental Cell Research. 2010May1;316(8):1324–31.

127. Nara Y, Kato Y, Torii Y, Tsuji Y, Nakagaki S, Goto S, et al. Immunohistochemical localization of extracellular matrix components in human breast tumours with special reference to PG-M/versican. Histochem. J. 1997Feb.20;29(1):21–30.

128. Yamaguchi Y, Ruoslahti E. Expression of human proteglycan in chinese hamster ovary cells inhibits cell proliferation. Nature. 1988;336:281–4.

129. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. nature. 1990Jul. 19;346(6281):281–4.

130. De Luca A, Santra M, Baldi A, Giordano A, Iozzo RV. Decorin-induced growth suppression is associated with up-regulation of p21, an inhibitor of cyclin-dependent kinases. J. Biol. Chem. 1996Aug.2;271(31):18961–5.

131. Neill T, Schaefer L, Iozzo RV. Decorin: a guardian from the matrix. The American Journal of Pathology. 2012Aug.;181(2):380–7.

132. Zhu J-X, Goldoni S, Bix G, Owens RT, McQuillan DJ, Reed CC, et al. Decorin evokes protracted internalization and degradation of the epidermal growth factor receptor via caveolar endocytosis. J. Biol. Chem. 2005Sep.16;280(37):32468–79.

133. Santra M, Reed CC, Iozzo RV. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J. Biol. Chem. 2002Sep.20;277(38): 35671–81.

134. Seidler DG, Goldoni S, Agnew C, Cardi C, Thakur ML, Owens RT, et al. Decorin protein core inhibits in vivo cancer growth and metabolism by hindering epidermal growth factor receptor function and triggering apoptosis via caspase-3 activation. J. Biol. Chem. 2006Sep.8;281(36): 26408–18.

135. Goldoni S, Humphries A, Nyström A, Sattar S, Owens RT, McQuillan DJ, et al. Decorin is a novel antagonistic ligand of the Met receptor. The Journal of Cell Biology. 2009May18;185(4):743–54.

136. Buraschi S, Pal N, Tyler-Rubinstein N, Owens RT, Neill T, Iozzo RV. Decorin antagonizes Met receptor activity and down-regulates {beta}-catenin and Myc levels. Journal of Biological Chemistry. 2010Dec. 31;285(53):42075–85.

137. Merline R, Moreth K, Beckmann J, Nastase MV, Zeng-Brouwers J, Tralhão JG, et al. Signaling by the matrix proteoglycan decorin controls inflammation and cancer through PDCD4 and MicroRNA-21. Sci Signal. 2011;4(199):ra75.

138. Ricciardelli C, Sakko AJ, Ween MP, Russell DL, Horsfall DJ. The biological role and regulation of versican levels in cancer. Cancer Metastasis Rev. 2009Jan.22;28(1-2):233–45.

139. Creighton CJ, Bromberg-White JL, Misek DE, Monsma DJ, Brichory F, Kuick R, et al. Analysis of tumor-host interactions by gene expression profiling of lung adenocarcinoma xenografts identifies genes involved in tumor formation. Mol. Cancer Res. 2005Mar.;3(3):119–29.

140. Ricciardelli C, Russell DL, Ween MP, Mayne K, Suwiwat S, Byers S, et al. Formation of hyaluronan- and versican-rich pericellular matrix by prostate cancer cells promotes cell motility. J. Biol. Chem. 2007Apr. 6;282(14):10814–25.

141. Gao D, Joshi N, Choi H, Ryu S, Hahn M, Catena R, et al. Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. Cancer Research. 2012Mar.15;72(6): 1384–94.

142. Grigoriu B-D, Depontieu F, Scherpereel A, Gourcerol D, Devos P, Ouatas T, et al. Endocan Expression and Relationship with Survival in Human Non-Small Cell Lung Cancer. Clin. Cancer Res. 2006Aug.1;12(15):4575–82.

143. Huang G-W, Tao Y-M, Ding X. Endocan expression correlated with poor survival in human hepatocellular carcinoma. Dig. Dis. Sci. 2009Feb.; 54(2):389–94.

144. Leroy X, Aubert S, Zini L, Franquet H, Kervoaze G, Villers A, et al. Vascular endocan (ESM-1) is markedly overexpressed in clear cell renal cell carcinoma. Histopathology. 2010Jan.;56(2):180–7.

145. Sarrazin S, Lyon M, Deakin JA, Guerrini M, Lassalle P, Delehedde M, et al. Characterization and binding activity of the chondroitin/dermatan sulfate chain from Endocan, a soluble endothelial proteoglycan. Glycobiology. 2010Nov.;20(11):1380–8.

146. Bechard D, Gentina T, Delehedde M, Scherpereel A, Lyon M, Aumercier M, et al. Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. J. Biol. Chem. 2001Dec.21;276(51):48341–9.

147. Scherpereel A, Gentina T, Grigoriu B, Sénéchal S, Janin A, Tsicopoulos A, et al. Overexpression of endocan induces tumor formation. Cancer Research. 2003Sep.15;63(18):6084–9.

148. Gu X, Ma Y, Xiao J, Zheng H, Song C, Gong Y, et al. Up-regulated biglycan expression correlates with the malignancy in human colorectal cancers. Clin. Exp. Med. 2012Sep.;12(3):195–9.

149. Wang B, Li G-X, Zhang S-G, Wang Q, Wen Y-G, Tang H-M, et al. Biglycan expression correlates with aggressiveness and poor prognosis of gastric cancer. Experimental Biology and Medicine. 2011Nov.1;236(11):1247–53.

150. Weber CK, Sommer G, Michl P, Fensterer H, Weimer M, Gansauge F, et al. Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. Gastroenterology. 2001Sep.;121(3):657–67.

151. Tanaka K, Arao T, Tamura D, Aomatsu K, Furuta K, Matsumoto K, et al. SRPX2 Is a Novel Chondroitin Sulfate Proteoglycan That Is Overexpressed in Gastrointestinal Cancer. Algül H, editor. PLoS ONE. 2012Jan.5;7(1):e27922.

152. Price MA, Colvin Wanshura LE, Yang J, Carlson J, Xiang B, Li G, et al. CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. Pigment Cell & Melanoma Research. 2011Dec.;24(6):1148–57.

153. Yang J, Price MA, Li GY, Bar-Eli M, Salgia R, Jagedeeswaran R, et al. Melanoma Proteoglycan Modifies Gene Expression to Stimulate Tumor Cell Motility, Growth, and Epithelial-to-Mesenchymal Transition. Cancer Research. 2009Oct.1;69(19):7538–47.

154. Marhaba R, Zöller M. CD44 in cancer progression: adhesion, migration and growth regulation. J. Mol. Histol. 2004Mar.;35(3):211–31.

155. Zöller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer. 2011Apr.;11(4):254–67.

156. Lamontagne C-A, Grandbois M. PKC-induced stiffening of hyaluronan/ CD44 linkage; local force measurements on glioma cells. Experimental Cell Research. 2008Jan.15;314(2):227–36.

157. Oliferenko S, Kaverina I, Small JV, Huber LA. Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. The Journal of Cell Biology. 2000Mar.20;148(6):1159–64.

158. van der Voort R, Taher TE, Wielenga VJ, Spaargaren M, Prevo R, Smit L, et al. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/ scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. J. Biol. Chem. 1999Mar.5;274(10):6499–506.

159. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H. CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes & Development. 2002Dec.1;16(23):3074–86.

160. Orian-Rousseau V, Morrison H, Matzke A, Kastilan T, Pace G, Herrlich P, et al. Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. Mol. Biol. Cell. 2007Jan.; 18(1):76–83.

161. Maeda N, Ishii M, Nishimura K, Kamimura K. Functions of chondroitin sulfate and heparan sulfate in the developing brain. Neurochem. Res. 2011Jul.;36(7):1228–40.

162. Crespo D, Asher RA, Lin R, Rhodes KE, Fawcett JW. How does chondroitinase promote functional recovery in the damaged CNS? Exp. Neurol. 2007Aug.;206(2):159–71.

163. Mitsunaga C, Mikami T, Mizumoto S, Fukuda J, Sugahara K. Chondroitin sulfate/dermatan sulfate hybrid chains in the development of cerebellum. Spatiotemporal regulation of the expression of critical disulfated disaccharides by specific sulfotransferases. J. Biol. Chem. 2006Jul. 14;281(28):18942–52.

164. Akatsu C, Mizumoto S, Kaneiwa T, Maccarana M, Malmström A, Yamada S, et al. Dermatan sulfate epimerase 2 is the predominant isozyme in the formation of the chondroitin sulfate/dermatan sulfate hybrid structure in postnatal developing mouse brain. Glycobiology. 2011Apr.6;21(5):565–74.

165. Bao X, Pavão MSG, Santos Dos JC, Sugahara K. A functional dermatan sulfate epitope containing iduronate(2-O-sulfate)alpha1-3GalNAc(6-O-sulfate) disaccharide in the mouse brain: demonstration using a novel monoclonal antibody raised against dermatan sulfate of ascidian Ascidia nigra. J. Biol. Chem. 2005Jun.17;280(24):23184–93.

166. Bao X, Nishimura S, Mikami T, Yamada S, Itoh N, Sugahara K. Chondroitin sulfate/dermatan sulfate hybrid chains from embryonic pig brain, which contain a higher proportion of L-iduronic acid than those from adult pig brain, exhibit neuritogenic and growth factor binding activities. J. Biol. Chem. 2004Mar.12;279(11):9765–76.

167. Hikino M, Mikami T, Faissner A, Vilela-Silva A-CES, Pavão MSG, Sugahara K. Oversulfated dermatan sulfate exhibits neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons: implications of dermatan sulfate in neuritogenesis in the brain. J. Biol. Chem. 2003Oct.31;278(44):43744–54.

168. Perissinotto D, Iacopetti P, Bellina I, Doliana R, Colombatti A, Pettway Z, et al. Avian neural crest cell migration is diversely regulated by the two major hyaluronan-binding proteoglycans PG-M/versican and aggrecan. Development. 2000Jul.;127(13):2823–42.

169. Inatani M, Honjo M, Otori Y, Oohira A, Kido N, Tano Y, et al. Inhibitory effects of neurocan and phosphacan on neurite outgrowth from retinal ganglion cells in culture. Investigative Ophthalmology & Visual Science. 2001Jul.;42(8):1930–8.

170. Seidenbecher CI, Gundelfinger ED, Böckers TM, Trotter J, Kreutz MR. Transcripts for secreted and GPI-anchored brevican are differentially distributed in rat brain. Eur. J. Neurosci. 1998May;10(5):1621–30.

171. Milev P, Friedlander DR, Sakurai T, Karthikeyan L, Flad M, Margolis RK, et al. Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. The Journal of Cell Biology. 1994Dec.;127(6 Pt 1):1703–15.

172. Milev P, Monnerie H, Popp S, Margolis RK, Margolis RU. The core protein of the chondroitin sulfate proteoglycan phosphacan is a high-affinity ligand of fibroblast growth factor-2 and potentiates its mitogenic activity. J. Biol. Chem. 1998Aug.21;273(34):21439–42.

173. Milev P, Chiba A, Häring M, Rauvala H, Schachner M, Ranscht B, et al. High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule. J. Biol. Chem. 1998Mar.20;273(12):6998–7005.

174. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. Cell. 1997Aug.22;90(4):753–62.

175. Tsuchida K, Shioi J, Yamada S, Boghosian G, Wu A, Cai H, et al. Appican, the proteoglycan form of the amyloid precursor protein, contains chondroitin sulfate E in the repeating disaccharide region and 4-O-sulfated galactose in the linkage region. J. Biol. Chem. 2001Oct. 5;276(40):37155–60.

176. Umehara Y, Yamada S, Nishimura S, Shioi J, Robakis NK, Sugahara K. Chondroitin sulfate of appican, the proteoglycan form of amyloid precursor protein, produced by C6 glioma cells interacts with heparin-binding neuroregulatory factors. FEBS Lett. 2004Jan.16;557(1-3):233–8.

177. Bonnet F, Perin JP, Charbonnier F, Camuzat A, Roussel G, Nussbaum JL, et al. Structure and cellular distribution of mouse brain testican. Association with the postsynaptic area of hippocampus pyramidal cells. J. Biol. Chem. 1996Feb.23;271(8):4373–80.

178. Maccarana M, Olander B, Malmström J, Tiedemann K, Aebersold R, Lindahl U, et al. Biosynthesis of dermatan sulfate: chondroitin-glucuronate C5-epimerase is identical to SART2. J. Biol. Chem. 2006Apr. 28;281(17):11560–8.

179. Pacheco B, Malmström A, Maccarana M. Two dermatan sulfate epimerases form iduronic acid domains in dermatan sulfate. J. Biol. Chem. 2009Apr. 10;284(15):9788–95.

180. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J. Immunol. 2000Mar.1;164(5):2565–74.

181. Catlow KR, Deakin JA, Wei Z, Delehedde M, Fernig DG, Gherardi E, et al. Interactions of hepatocyte growth factor/scatter factor with various glycosaminoglycans reveal an important interplay between the presence of iduronate and sulfate density. J. Biol. Chem. 2008Feb.29;283(9):5235–48.

182. Kwok JCF, Afshari F, García-Alías G, Fawcett JW. Proteoglycans in the central nervous system: plasticity, regeneration and their stimulation with chondroitinase ABC. Restor. Neurol. Neurosci. 2008;26(2-3):131–45.

183. Fraser PE, Darabie AA, McLaurin JA. Amyloid-beta interactions with chondroitin sulfate-derived monosaccharides and disaccharides. implications for drug development. J. Biol. Chem. 2001Mar.2;276(9): 6412–9.

184. Goossens D, Van Gestel S, Claes S, De Rijk P, Souery D, Massat I, et al. A novel CpG-associated brain-expressed candidate gene for chromosome 18q-linked bipolar disorder. Mol Psychiatry. 2003;8(1):83–9.

185. Penc SF, Pomahac B, Winkler T, Dorschner RA, Eriksson E, Herndon M, et al. Dermatan sulfate released after injury is a potent promoter of fibroblast growth factor-2 function. J. Biol. Chem. 1998Oct.23;273(43):28116–21.

186. Dam ten GB, Yamada S, Kobayashi F, Purushothaman A, van de Westerlo EMA, Bulten J, et al. Dermatan sulfate domains defined by the novel antibody GD3A12, in normal tissues and ovarian adenocarcinomas. Histochem Cell Biol. 2009Jul.;132(1):117–27.

187. Petricevich VL, Michelacci YM. Proteoglycans synthesized in vitro by nude and normal mouse peritoneal macrophages. Biochim. Biophys. Acta. 1990Jul.12;1053(2-3):135–43.

188. Taylor KR, Rudisill JA, Gallo RL. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. J. Biol. Chem. 2005Feb.18;280(7):5300–6.

189. Terasaki M, Shibui S, Narita Y, Fujimaki T, Aoki T, Kajiwara K, et al. Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen--A24 with recurrent or progressive glioblastoma multiforme. J. Clin. Oncol. 2011Jan.20;29(3):337–44.

190. Noguchi M, Kobayashi K, Suetsugu N, Tomiyasu K, Suekane S, Yamada A, et al. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate. 2003Sep.15;57(1):80–92.

191. Schmidt HH, Dyomin VG, Palanisamy N, Itoyama T, Nanjangud G, Pirc-Danoewinata H, et al. Deregulation of the carbohydrate (chondroitin 4) sulfotransferase 11 (CHST11) gene in a B-cell chronic lymphocytic leukemia with a t(12;14)(q23;q32). Oncogene. 2004Sep.9;23(41):6991–6.

192. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012Feb.;12(2):89–103.

193. Cöster L, Hernnäs J, Malmström A. Biosynthesis of dermatan sulphate proteoglycans. The effect of beta-D-xyloside addition on the polymer-modification process in fibroblast cultures. Biochem. J. 1991Jun.1;276 (Pt 2):533–9.

194. Sugahara K, Mikami T. Chondroitin/dermatan sulfate in the central nervous system. Current Opinion in Structural Biology. 2007Oct.;17(5):536–45.

195. Rauch U, Kappler J. Chondroitin/Dermatan sulfates in the central nervous system: their structures and functions in health and disease. Adv. Pharmacol. 2006;53:337–56.

196. Kwok JCF, Warren P, Fawcett JW. Chondroitin sulfate: A key molecule in the brain matrix. International Journal of Biochemistry and Cell Biology. Elsevier Ltd; 2012Apr.1;44(4):582–6.

197. Irie F, Badie-Mahdavi H, Yamaguchi Y. Autism-like socio-communicative deficits and stereotypies in mice lacking heparan sulfate. Proc. Natl. Acad. Sci. U.S.A. 2012Mar.27;109(13):5052–6.

198. Duman RS, Monteggia LM. A neurotrophic model for stress-related mood disorders. Biological Psychiatry. 2006Jun.15;59(12):1116–27.

199. Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J. Neurosci. 1995Mar.;15(3 Pt 1):1768–77.

200. Sherman L, Wainwright D, PONTA H, Herrlich P. A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. Genes & Development. 1998Apr.1;12(7):1058–71.

201. Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, et al. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J. Cell. Physiol. 2000Jul.;184(1):118–30.