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Alexander Birbrair *Editor*

Tumor Microenvironment

Extracellular Matrix Components – Part B

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Tumor Microenvironment

Extracellular Matrix Components – Part B

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This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of Mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life I owe to her.

My beloved mom Marina Sobolevsky of blessed memory (July 28, 1959 – June 3, 2020).

Preface

This book's initial title was "Tumor Microenvironment." However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book "Tumor Microenvironment: Extracellular Matrix Components – Part B" presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different extracellular matrix components in the tumor microenvironment during cancer development. Further insights into these mechanisms will have important implications for our understanding of cancer initiation, development, and progression. The authors focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of the tumor microenvironment in several tissues using state-of-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of tumor biology, which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the extracellular matrix components in the tumor microenvironment in various tissues. Nine chapters written by experts in the field summarize the present knowledge about distinct extracellular matrix constituents during tumor development.

Murray B. Resnick and colleagues from Brown University discuss Elastin in the tumor microenvironment. Kornélia Baghy and colleagues from Semmelweis University describe Decorin in the tumor microenvironment. Adriana Handra-Luca from Universite Paris Nord Sorbonne updates us with what we know about Syndecan-1 in the tumor microenvironment. Athanasios Papadas and Fotis Asimakopoulos from University of California San Diego address the importance of Versican in the tumor microenvironment. Marta Mellai and colleagues from Università del Piemonte Orientale compile our understanding of Chondroitin Sulphate Proteoglycans in the tumor microenvironment. Andras G. Lacko and colleagues from The University of Texas Health Science Center at Fort Worth summarize current knowledge on

Lipoproteins in the tumor microenvironment. Kristin A. Giamanco and Russell T. Matthews from SUNY Upstate Medical University talk about the role of BEHAB/Brevican in the glioma microenvironment. Divya Ramchandani and Vivek Mittal from Weill Cornell Medicine focus on the effect of Thrombospondin in the tumor microenvironment. Finally, Shuli Xia and colleagues from Johns Hopkins School of Medicine give an overview of the Tenascin-C function in glioma.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife Veranika Ushakova and Mr. Murugesan Tamilsevan from Springer, who helped at every step of the execution of this project.

New York, NY, USA Alexander Birbrair
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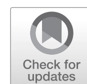

1 Elastin in the Tumor Microenvironment

Yihong Wang, Elizabeth C. Song, and Murray B. Resnick

Abstract

Elastic fibers are found in the extracellular matrix (ECM) of tissues requiring resilience and depend on elasticity. Elastin and its degradation products have multiple roles in the oncologic process. In many malignancies, the remodeled ECM expresses high levels of the elastin protein which may have either positive or negative effects on tumor growth. Elastin cross-linking with other ECM components and the enzymes governing this process all have effects on tumorigenesis. Elastases, and specifically neutrophil elastase, are key drivers of invasion and metastasis and therefore are important targets for inhibition. Elastin degradation leads to the generation of bioactive fragments and elastin-derived peptides that further modulate tumor growth and spread. Interestingly, elastin-like peptides (ELP) and elastin-derived peptides (EDP) may also be utilized as nano-carriers to combat tumor growth. EDPs drive tumor development in a variety of ways, and specifically targeting EDPs and their binding proteins are major objectives for ongoing and future anti-cancer therapies. Research on both the direct anti-cancer activity and the drug delivery capabilities of ELPs is another area likely to result in novel therapeutic agents in the near future.

Keywords

Elastin · Elastic fiber · Elastin-binding protein · Elastosis · Elastoma · Extracellular matrix · Elastase · Neutrophil elastase · Elastin-like peptide · Elastin-derived peptide · Elastin receptor · Tumor-associated stroma · Lysyl oxidases · Elastin collagen cross-linking · Tumor microenvironment

1.1 Introduction

Elastin, one of the longest-lived proteins, is a major component of the extracellular matrix and confers flexibility to tissues requiring mechanical resilience. Elastin and its degradation products have multiple roles in the oncologic process. In many malignancies, the remodeled extracellular matrix (ECM) expresses high levels of the elastin protein which may have either positive or nega-

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tive effects on tumor growth. Elastin cross-linking with other ECM components and the enzymes governing this process all have effects on tumorigenesis. During tumor progression, elastin in the ECM is enzymatically degraded allowing for local tumor spread and metastasis. Neutrophil elastase is one of the key and best studied elastases governing this process. Elastin degradation leads to the generation of bioactive fragments and elastin-derived peptides that further modulate tumor growth and spread. Interestingly, elastin-like peptides may also be utilized as nano-carriers to combat tumor growth. These topics will be expanded upon in the following chapters.

1.2 Elastin Fibers and Elastin Synthesis

Elastic fibers are extracellular matrix components that endow connective tissue resilience and are present in large proportions in tissues requiring mechanical pliability such as the arteries, skin, lungs, and cartilage [1–3]. Elastic fibers are made up of two key components: (1) elastin, an amorphous protein that constitutes the fiber core, and (2) microfibrils made up of glycoproteins which surround the core [1, 4] (Fig. 1.1).

The fundamental building block of elastin is the soluble monomeric protein tropoelastin. Humans have only one tropoelastin gene (*ELN*) [5]. The *ELN* gene is expressed during prenatal and postnatal life for the first few years. Its expression then drops to near-complete repression at maturity [6, 7]. Mature tropoelastin associates with the elastin-binding protein (EBP) intracellularly, and the tropoelastin-EBP complex is transported to the cell surface and secreted out of cells [8], where competition from extracellular galactosides results in the dissociation of tropoelastin from EBP; the latter is recycled back inside the cell [3] (Fig. 1.1). The *ELN* gene transcripts are subject to extensive alternative splicing which give rise to a variety of tropoelastin isoforms [9].

The tropoelastin released on the cell surface subsequently aggregates and deposits onto microfibrils, composed of glycoproteins such as fibrillin-1 and fibrillin-2, microfibril-associated glycoprotein-1, EMILINs, latent transforming growth factor beta binding proteins, and others [10]. Microfibrils serve as a scaffold to direct tropoelastin alignment, cross-linking, and consequential elastic fiber formation [11]. Lysyl oxidase (LOX) deaminates lysine residues to form allysine, which reacts with adjacent allysine or lysine to form cross-links [12]. Further reaction can lead to two major amino acids in elastin – desmosine and isodesmosine cross-links between tropoelastin molecules [13]. Cross-links within and between adjacent tropoelastin molecules result in the mature insoluble elastic fiber, which is a hydrophobic durable polymer, that is resistant to enzymatic proteolysis and experiences essentially no turnover in healthy tissues [14].

1.3 Tumor Elastosis

Stromal elastosis, defined as dense aggregates of elastic fibers, is a pathological stromal alteration seen in neoplastic tissue stroma. Tumor elastosis was first described in breast cancer by Cheatle and Cutler in 1931 [15], and the term elastosis was later coined by Jackson and Orr [16]. Elastosis has also been historically described as “scirrhous,” “chalky streaks” [17], and “amyloid” [18]. Elastosis is eosinophilic, pale, and homogeneous on the hematoxylin and eosin (H&E) stain and can be distinctively highlighted on elastic-van Gieson’s (EVG) stain and immunohistochemically using anti-elastin antibodies (Fig. 1.2).

1.3.1 Elastosis in Breast Cancer

Breast cancer has been the most comprehensively studied of all malignancies exhibiting elastosis. The first detailed morphological description of elastosis in breast cancer was provided by Lundmark and by Shivas et al. in 1972 [19, 20]. In 1974, Azzopardi and Laurini described two types of elastosis occurring in breast cancer: peri-

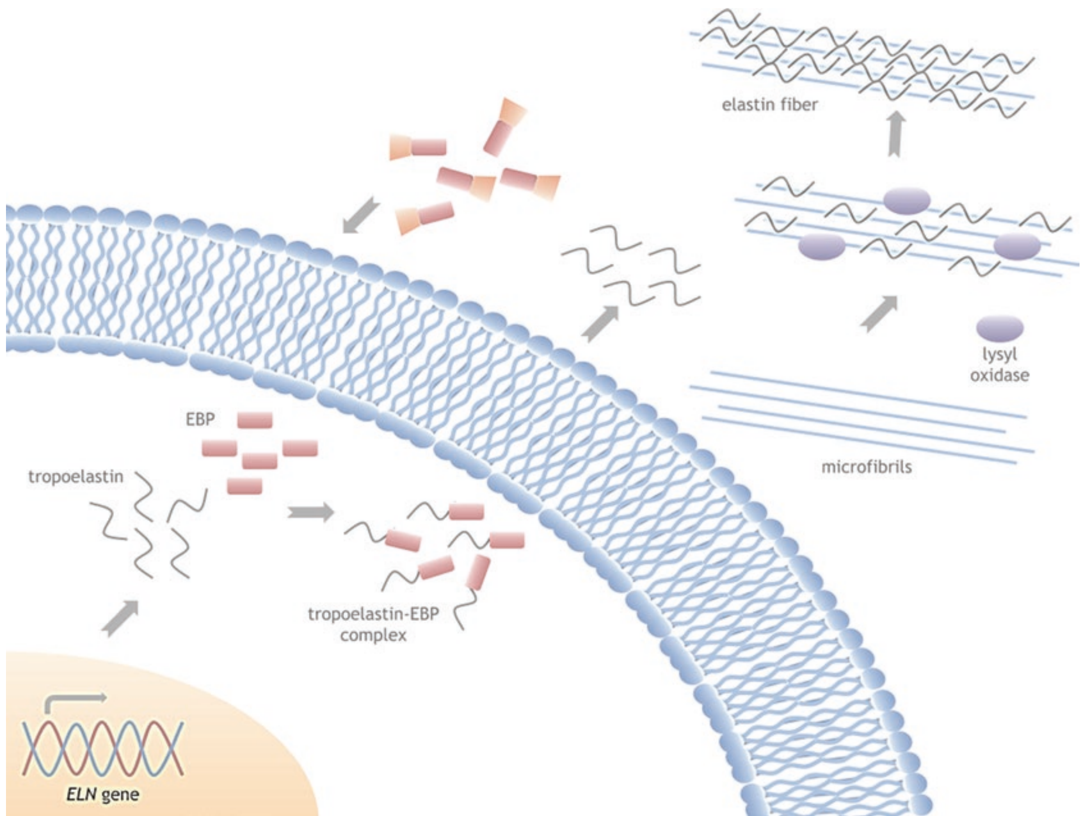

Fig. 1.1 Elastin biosynthesis. Tropoelastin is transcribed and translated from the elastin (ELN) gene and transported to the plasma membrane in association with elastin-binding protein (EBP) to prevent its aggregation and premature degradation. EBP-tropoelastin assembly is then directed to the plasma membrane. EBP is secreted, disassociates from the complex, and binds to galactose sugars and recycles back into the cell nucleus. Tropoelastin is released and aggregates on the cell surface. Tropoelastin aggregates are oxidized by lysyl oxidase leading to cross-linked elastin that accumulates on microfibrils which help to direct elastin deposition. The process of deposition and cross-linking continues to give rise to mature elastic fibers

ductal and vascular. They described elastosis occurring in 90% of infiltrating breast carcinomas and postulated that breast cancer cells secreted factors which induced fibroblasts to produce elastin [21]. They further suggested that elastosis may be an early indicator of tumor invasion. This concept was elaborated on by Lundmark who maintained that elastosis accompanying ductal carcinoma in situ is an indicator of early invasion and correlates with age, tumor type, and grade [19]. The degree of periductal elastosis and stromal elastosis increases progressively with the severity of breast disease [22, 23], and several studies have correlated elastin with estrogen receptor content [24–28].

The prognostic significance of elastosis in breast cancer is unclear [20, 25, 29]. Rasmussen et al. failed to demonstrate any prognostic significance of elastosis in a group of 171 primary breast carcinomas [30]. In a more recent study, Chen et al. established that the presence of elastosis was associated with low tumor cell proliferation and a good prognosis [31]. Masters et al. found a positive correlation between tumor elastosis and endocrine therapy response [32].

It has been suggested that the source of elastic fibers in breast cancer may result from neoplastic

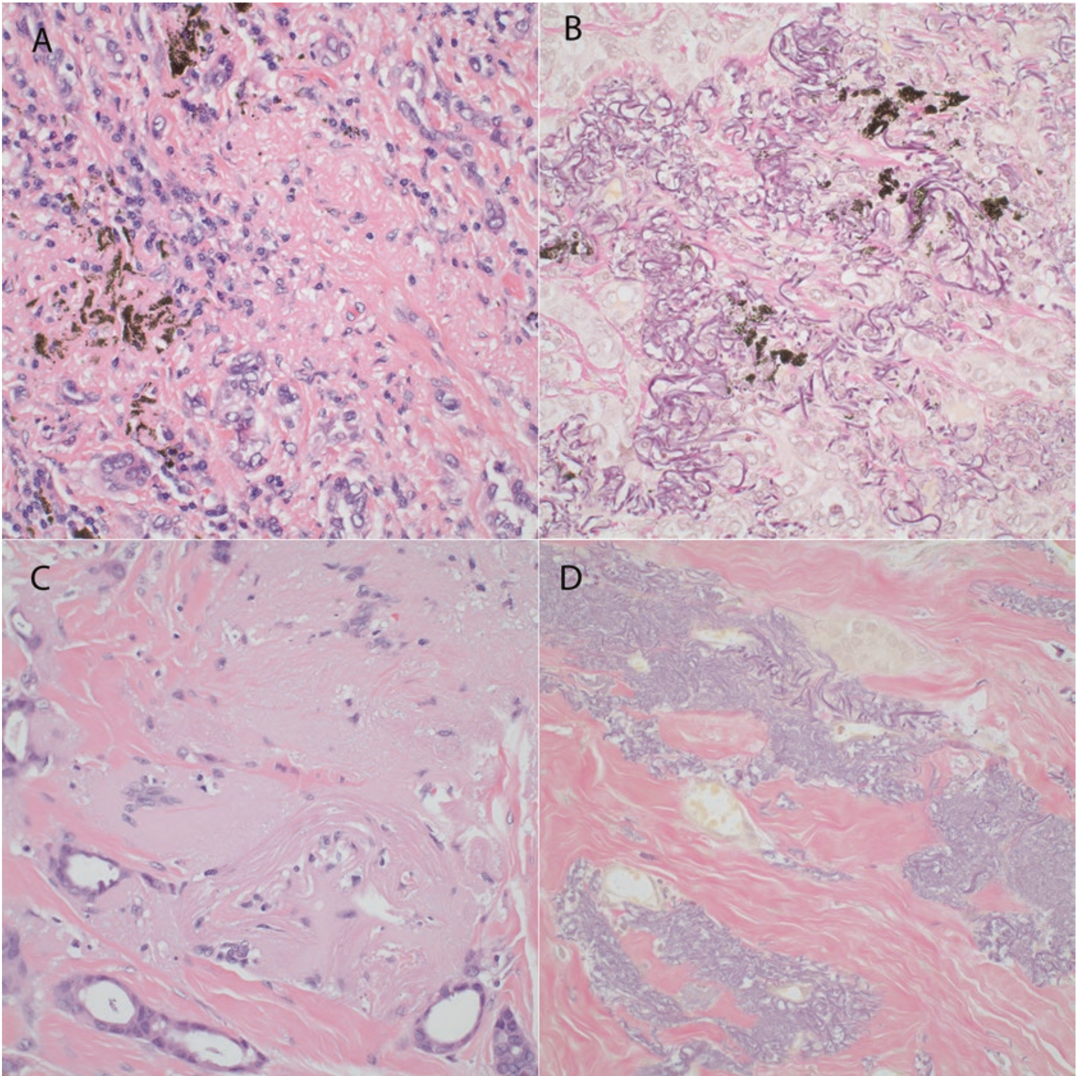

Fig. 1.2 Cancer-associated stromal response with prominent elastosis. (**a** and **b**) Breast cancer H&E and elastic stain; (**c** and **d**) lung cancer H&E and elastic stain. (Magnification 400×)

epithelial cells provoking stromal fibroblasts [21, 23, 33, 34] or from the neoplastic epithelial cells themselves [20, 35]. In a largely immunohistochemical study, Mera et al. identified elastic fibers in breast cancer stroma [36]. Using mRNA in situ hybridization, Krishnan and Cleary demonstrated that in some invasive breast cancers, stromal cells are the source of elastotic materials, whereas in others the malignant epithelial cells are responsible [37]. Breast cancer cell line studies further confirmed that tumor cells are capable of producing tropoelastin [37, 38].

1.3.2 Elastosis in Other Solid Tumors

Elastosis has been also been described in tumor-associated stroma of the lung, salivary gland, thyroid, cervix, stomach, and prostate [39–42].

Elastosis in lung carcinomas is more commonly seen in adenocarcinomas than squamous cell carcinomas and was not detected in small-cell or large-cell carcinomas [43]. Elastosis in pulmonary adenocarcinomas was more commonly associated with well-differentiated tumors and was associated with better patient outcome in low-stage tumors [43]. Azzopardi and Zayid first described the association of elastosis with salivary gland tumors [44], and David et al. described the presence of elastosis in pleomorphic adenomas, malignant pleomorphic adenomas, and adenoid cystic neoplasms of the salivary glands but not in other variants of salivary gland tumors [45]. Elastosis is identified more frequently with classical papillary thyroid carcinomas than the follicular variant. In papillary carcinomas, deposits of elastic fibers vary in size and shape and are more frequently distributed at the periphery of the tumor tissue [41].

1.4 Elastin-Derived Peptides

The functional form of elastin is a highly cross-linked polymer that organizes as sheets or fibers in the extracellular matrix. The fact that purified elastin remains insoluble [46] severely limits its use in biological experiments. As a consequence, elastin-derived peptides (EDP) have been widely used, allowing considerable advances in the understanding of elastin fiber aging and remodeling. The term "elastin peptides" designates both enzymatically and chemically produced peptides. The former are the results of elastin digestion by elastases. They are often termed elastin lysate. The latter corresponds either to synthetic peptides or to the peptide mixture obtained after mild chemical hydrolysis of insoluble elastin by oxalic acid [47]. Elastin is highly resistant to proteolysis and experiences essentially no turnover in normal physiological conditions. However, members of the serine, aspartate, and cysteine proteases and matrix metalloproteinases (MMP) superfamilies can fragment elastin to elastin peptides during several pathological and physiopathological processes [48–50]. The elastin peptides generated during elastin degradation can modulate the behavior of a broad range of biological activities including chemotaxis, proliferation, and protease release. In cancers, elastin peptides have been shown to modulate the cellular physiology of tumor cells, stromal fibroblasts, smooth muscle cell, endothelial cells, and inflammatory cells [51–54].

The chemotactic effect of elastin peptides has been mainly studied in monocytes. The κ-elastin induces an increase of intracellular cyclic 3,5_-adenosine monophosphate (cAMP), cyclic 3,5_-guanosine monophosphate (cGMP), and Ca^{2+} levels and stimulates the respiratory burst in monocytes; the authors suggested the increased cGMP level related to chemotaxis. The effect of elastin peptides on intracellular calcium level and cGMP levels plays a role in the modifications of the extracellular matrix following elastin degradation as observed in atherosclerosis [55, 56]. A study by Pocza et al. found that elastic peptides have a potent chemotactic effect on melanoma cells and their presence at a distant organ might contribute to metastasis [57]. Recently, elastin peptides have been linked to in vivo regulation of tumor progression of melanoma, which also has an elastin-rich tumor-associated stroma [58].

The signaling pathways related to elastin peptide-induced proliferation have been recently studied in porcine arterial smooth muscle cells [59]. The authors have shown that elastin hydrolysates and synthetic peptides trigger signaling pathways leading to the opening of L-type calcium channels and activation of pertussis toxin-sensitive G proteins. Moreover, they point out that c-Src, the Ras/Raf/MEK/ERK signaling cascade, and the platelet-derived growth factor receptor are also involved in elastin peptide-induced proliferation. EDPs induced in vitro proliferation have been shown in several neoplastic cell lines including glioma, astrocytoma, and melanoma [60]. Surface aggregation of elastin receptor molecules caused by suramin amplified signals leads to proliferation of human glioma cells. Further evidence for EDP-induced proliferative potential can be seen in tropoelastin and EDPs, which promote proliferation of human astrocytoma cell lines [61, 62] are additional evidences of EDPs induced proliferative potential.

The synthesis of proteases such as matrix metalloproteinases (MMP) by tumor and stroma cells during stromal reaction is one of the crucial events leading to matrix degradation and tumor progression [63]. Elastin peptides upregulate pro-MMP-1 production in skin fibroblasts and HT-1080 tumor cells [64, 65]. EDPs enhance melanoma growth in vivo by upregulating the activation of Mcol-A(MMP-1) collagenase [62]. In addition, Toupance et al. demonstrated that elastin peptides stimulate lung cancer cell invasion post-transcriptionally by regulation of MMP-2 and urokinase plasminogen activator [66].

1.4.1 Elastin-Derived Peptides and the Elastin Receptor

Elastin-derived peptides display a wide range of biological activity by interacting with the elastin receptor complex (ERC). The ERC is expressed on the surface of numerous cell types [67]. Three cell surface receptors have been described to mediate the effects of elastin peptides: ERC, aVb3 integrin, and galectin-3. ERC, the primary receptor, is comprised of three subunits: the elastin-binding protein (EBP), neuraminidase-1 (Neu-1), and the protective protein/cathepsin A (PPCA) [68–70].

The 67 kDa elastin-binding protein (EBP) is peripheral and binds the VGVAPG domain on tropoelastin with high affinity. It also possesses galactolectin properties and therefore has the ability to fix β-galactosugars such as galactose or lactose. EBP, identified as an enzymatically inactive form of β-galactosidase, is the subunit which actually binds the elastic peptide [71]. The two other subunits are membrane-associated, and the catalytic activity of Neu-1 is crucial for elastin receptor complex signaling [72]. Typical elastin receptor complex ligands are peptides containing the xGxxPG (where x represents any amino acid) consensus sequence thought to favor a local type VIII b-turn conformation [73]. This conformation has been linked to the bioactivity of the VGVAPG peptide [74, 75].

αVβ3 integrin is another cell surface receptor mediating elastin peptide signaling. Traditionally, integrins bind to extracellular matrix through RGD motifs [76]. However, there are several non-RGD sequences serving as ligands for integrins [77]. Recently, the aVb3 integrin has been reported to interact directly with tropoelastin and the elastin peptide to mediate their effects [78, 79]. The third elastin peptide receptor is galectin-3. Galectin-3 is a 31 kDa β-galactoside-binding lectin and plays an important role in cell-extracellular matrix interactions [80]. Its expression has been linked to tumor progression and cancer aggressiveness [81]. Galectin-3 is capable of regulating the interactions between cells and elastin and to bind the VGVAPG elastin peptide leading to melanoma invasion [79, 82].

1.4.2 Elastin-Like Polypeptides and Cancer Therapy

Identifying the nature of elastin-derived proteins' cryptic sequences and the characterization of their bioactive structures may be utilized for the conception of antagonists blocking the elastin-binding site. Elastin-like polypeptides (ELPs) are comprised of a genetically engineered class of molecules derived from tropoelastin. Such substances could have anti-cancer properties such as reducing cell proliferation, chemotactic response of malignant cells, and MMP synthesis as reviewed recently by Despanie et al. [83]. In addition to these cancer treatment applications, ELPs may represent a promising class of recombinant biopolymers for the delivery of drugs and imaging agents to solid tumors via systemic or local administration. Small hydrophobic drugs can be conjugated to the C-terminus of the elastin-like polypeptides to impart the amphiphilicity needed to mediate the self-assembly of nanoparticles [84]. These systemically delivered ELPs –drug nanoparticles – preferentially localize to the tumor site via the permeability and retention effect, resulting in reduced toxicity and enhanced treatment efficacy [85]. In order to improve the pharmacokinetic profiles of the ELP drug delivery platform, researchers have modi-

fied the system using genetically engineered ELP incorporated with multiple copies of the IL-4 receptor targeting peptide (AP1) and the pro-apoptotic peptide (KLAKLAK)2 referred to as AP1-ELP-KLAK. Systemic administration of AP1-ELP-KLAK significantly inhibited tumor growth by provoking cell apoptosis in various tumor xenograft models without any specific organ toxicity and also improved drug bioavailability, stability, membrane penetration, and drug half-life [85]. A newer application using a bladder tumor-targeting peptide – embedding ELP as a drug delivery vehicle – displayed excellent localization in bladder tumor-xenografted mice after intravenous injection and was strictly confined to specific antigen-overexpressing tumor tissue [86].

Other approaches to use elastin-like polypeptides in anti-cancer drug delivery take direct advantage of the thermal responsiveness of elastin-like polypeptides. At physiological temperatures, ELPs are entirely soluble, but at higher temperatures, they become insoluble by coacervation [87]. ELP-blocking copolymers may be designed to assemble into nanoparticles in response to hyperthermia due to the independent thermal transition of the hydrophobic block, thus resulting in multivalent ligand display of a ligand for spatially enhanced vascular targeting. Delivery of ELPs conjugated with radiotherapeutics maybe injected directly into tumor where they undergo coacervation to form an injectable drug depot for intratumoral delivery. These injectable coacervate ELP-radionuclide depots display a long residence in the tumor and result in inhibition of tumor growth [88].

1.5 Elastase and the Degradation of Elastin in Tumorigenesis

During invasion and metastasis, tumor cells confront a variety of natural tissue barriers in vivo, such as basement membranes and surrounding tissue stromal matrices including elastin. It is thus necessary for tumor cells to elaborate a battery of extracellular matrix degradative enzymes to achieve invasion and metastasis. Many different types of extracellular matrix degradative enzymes have been implicated in the invasive growth and metastasis of cancer cells [89–91]. Elastases are a heterogeneous group of enzymes capable of degrading mature, insoluble elastin protein under physiological conditions. They are found in most of the major proteolytic families, including serine, thiol, aspartic enzymes, and metalloenzymes [92]. There are three well-characterized mammalian elastases: (1) pancreatic elastase I, a serine protease secreted in zymogen form by pancreatic acinar cells; (2) neutrophil elastase, a neutral protease found in granules of human polymorphonuclear leukocytes [93, 94]; and (3) metalloprotease which is secreted by macrophages. Four metalloproteases (MMP-2, MMP-7, MMP-9, and MMP-12) are elastases [95]. Neutrophil elastase that belongs to the serine proteinase enzymes family exhibits the most potent proteolytic activity under physiological conditions and is also the most widely studied elastase.

Neutrophil elastase was first described as a serine protease stored in azurophilic granules of neutrophils. It is released into the extracellular space through degranulation or during neutrophil extracellular trap formation to carry out its physiological function of pathogen clearance during infection. Neutrophil elastase is also a critical regulator of the inflammatory response [96, 97]. Neutrophil elastase is implicated in matrix remodeling in a variety of pathological processes including chronic obstructive pulmonary disease [98], pulmonary fibrosis [99], and atherosclerosis [100]. During lung cancer progression, extensive destruction of the rich elastin network through the expression and activation of elastases is observed [101]. Elastinolytic activities in human breast cancer tissue have been demonstrated by Hornebeck et al. [102]. Thereafter, several investigators have described elastinolytic enzyme production by human and rodent mammary tumor cells [103–105]. In lung cancer patients, elevated serological neutrophil elastase positively correlates not only with disease state but also with disease progression [106]. Neutrophil elastase activity is three- and fivefold greater in the bron-

choalveolar lavage fluid and serum, respectively, in individuals with lung cancer compared to those with COPD [107]. Enhanced neutrophil elastase activity in lung cancer patients can also be detected indirectly through accumulation of neutrophil elastase-specific elastin degradation products [108]. A strong neutrophil elastase proteolytic fingerprint distinguishes the colon adenocarcinoma proteome from that of ulcerative colitis [109]. In breast cancer, high neutrophil elastase immunoreactivity is an independent poor prognosis factor correlated with diminished metastasis-free survival, relapse-free survival, and overall survival [110–112].

Neutrophil elastase is an integral component of neutrophil extracellular traps (NETs). Myeloid cells secrete neutrophil elastase and neutrophil extracellular traps (NETs) in response to cues within the tumor microenvironment, thereby leading to enhanced activity in cancer cells [113–115]. A recent study showed that DNA released from NETs activates pancreatic stellate cells and enhances pancreatic tumor growth [116]. Neutrophil elastase is likely a key driver of tumorigenesis and facilitator of metastasis, since genetic deletion and pharmacological inhibition markedly reduce tumor burden and metastatic potential in some studies [117–119]. Neutrophil elastase may therefore serve as a novel cancer biomarker or therapeutic target.

1.6 Elastin Collagen Cross-Linking

In the tumor microenvironment, fibrillar collagens represent the most abundant extracellular matrix proteins. Formation of covalent cross-links occurring between elastin and fibrillar collagen promotes the tensile strength of the ECM and is mediated by the action of lysyl oxidase (LOX) [120–123].

1.6.1 Lysyl Oxidases Role in Elastin Collagen Cross-Linking

While intracellular functions have been reported for LOX proteins, the primary role of this family of enzymes is the remodeling of the extracellular matrix. The best-studied role of LOX in the extracellular matrix is the cross-linking of collagens and elastin. The LOX family constitutes five members of extracellular copper-dependent amine oxidases including LOX and LOX-like isoforms (LOXL) 1–4 which are present in the extracellular matrix [reviews seen 124–125].

Collagen is the most abundant, naturally occurring protein in mammals. It is found in the bone, teeth, skin, ligaments, and tendons. Of the 27 naturally occurring collagen types, type I collagen is the most abundant type. Type I collagen is made up of two identical α1(I) chains and one α2(II) chain. These chains have a common repeating motif, Gly e XeY, where X and Y are primarily proline and hydroxyproline residues [126]. LOX catalyzes a key step in the cross-linking of collagen and elastin where lysine residues within the N- and C-terminal telopeptide regions are oxidized to aminoadipic d-semialdehyde [127]. These resulting aldehydes are condensed with unmodified lysine and hydroxylysine residues, creating cross-linkages. Examples of the cross-linking reactions are given in Fig. 1.3 [adapted from 124]. Elastin is also modified by LOX; these properties are important to the extracellular matrix because elastin must be able to adapt to its environment while at the same time retains its resilience. The mechanism for the formation of elastin cross-links is very similar to that of collagen, except that the cross-linking in elastin does not involve hydroxylysine, whereas desmosine and isodesmosine are not present in collagen [128]. Levental and colleagues showed that by modifying the state of collagen cross-linking and ECM stiffness, two physical parameters of the tissue microenvironment, the invasive behavior of an oncogene pre-transformed mammary epithelium could be modulated. This was characterized by promotion of focal adhesions, enhanced PI3 kinase (PI3K) activity, and induced invasion of an oncogene-

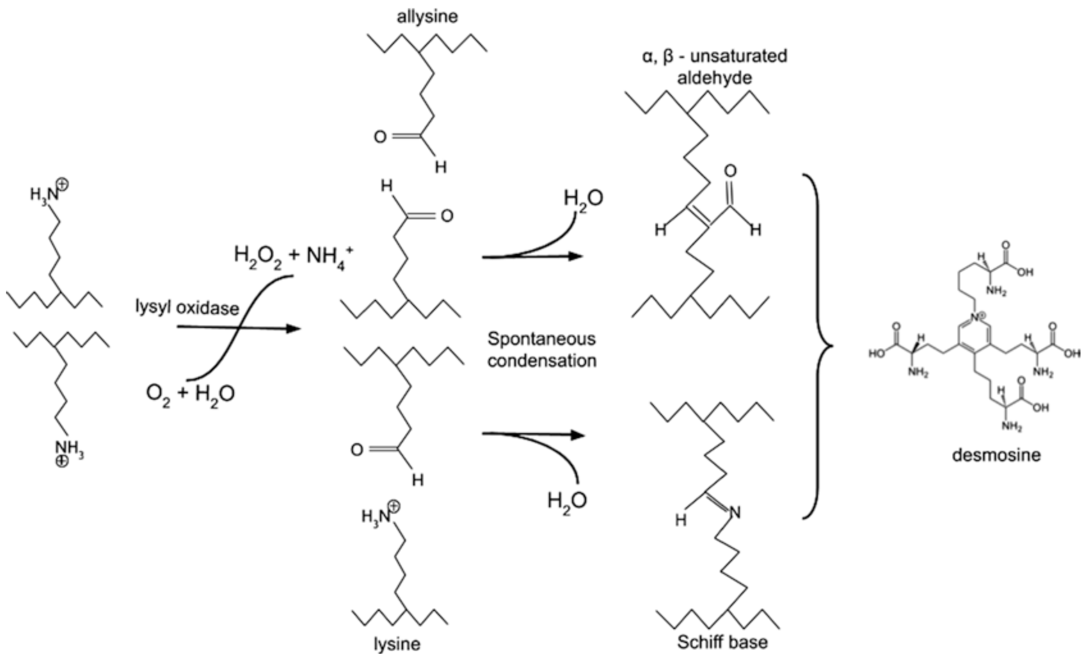

Fig. 1.3 Lysyl oxidase initiated cross-link formation in tropocollagen and tropoelastin. Lysyl oxidase catalyzes the oxidative deamination of lysine and hydroxylysine residues in tropocollagen and tropoelastin (only lysine residues are shown). The product allysine residues spontaneously react with other allysine or lysine residues via aldol condensation or Schiff base formation. The bifunctional condensation products can further cross-link to form tri-, tetra-, or even pentafunctional cross-links (depicted is desmosine, a common tetrafunctional cross-link found in elastin)

initiated epithelium, even in the absence of cellular and soluble tissue and systemic factors. The findings imply that changes of extracellular matrix-induced tissue fibrosis could regulate cancer behavior by influencing the biophysical properties of the microenvironment to alter force at the cell and/or tissue level [129].

LOX enzymes are widely expressed in tumor-associated extracellular matrix and possess a wide range of biological functions other than collagen/elastin cross-linking. During tumor development, tumor cells constantly communicate with the surrounding microenvironment to support tumor cell proliferation, epithelial-to-mesenchymal transition (EMT), migration, invasion, angiogenesis, and metastasis. Aberrant expression or activation of LOX alters the cellular microenvironment, leading to many diseases including atherosclerosis, tissue fibrosis, and cancer. This topic is somewhat removed from the scope of this chapter and was recently reviewed by Johnson and Lopez [125].

1.6.2 Collagen Elastin Cross-Linking in Tumorigenesis

Collagen type IX α-1 (ColXα1) is a short-chain collagen, typically found underlying endothelial cells and in the hypertrophic zone of cartilage during endochondral ossification where it participates in calcifying cartilage formation [12]. We have recently shown that increased ColXα1 predicts poor pathologic response in neoadjuvant-treated ER+/HER2+ breast tumors [130]. Interestingly we also observed that ColXα1 expression in breast tumors has a patchy distribution pattern reminiscent of elastosis. We further demonstrated using immunohistochemistry, immunofluorescence, and electron microscopy

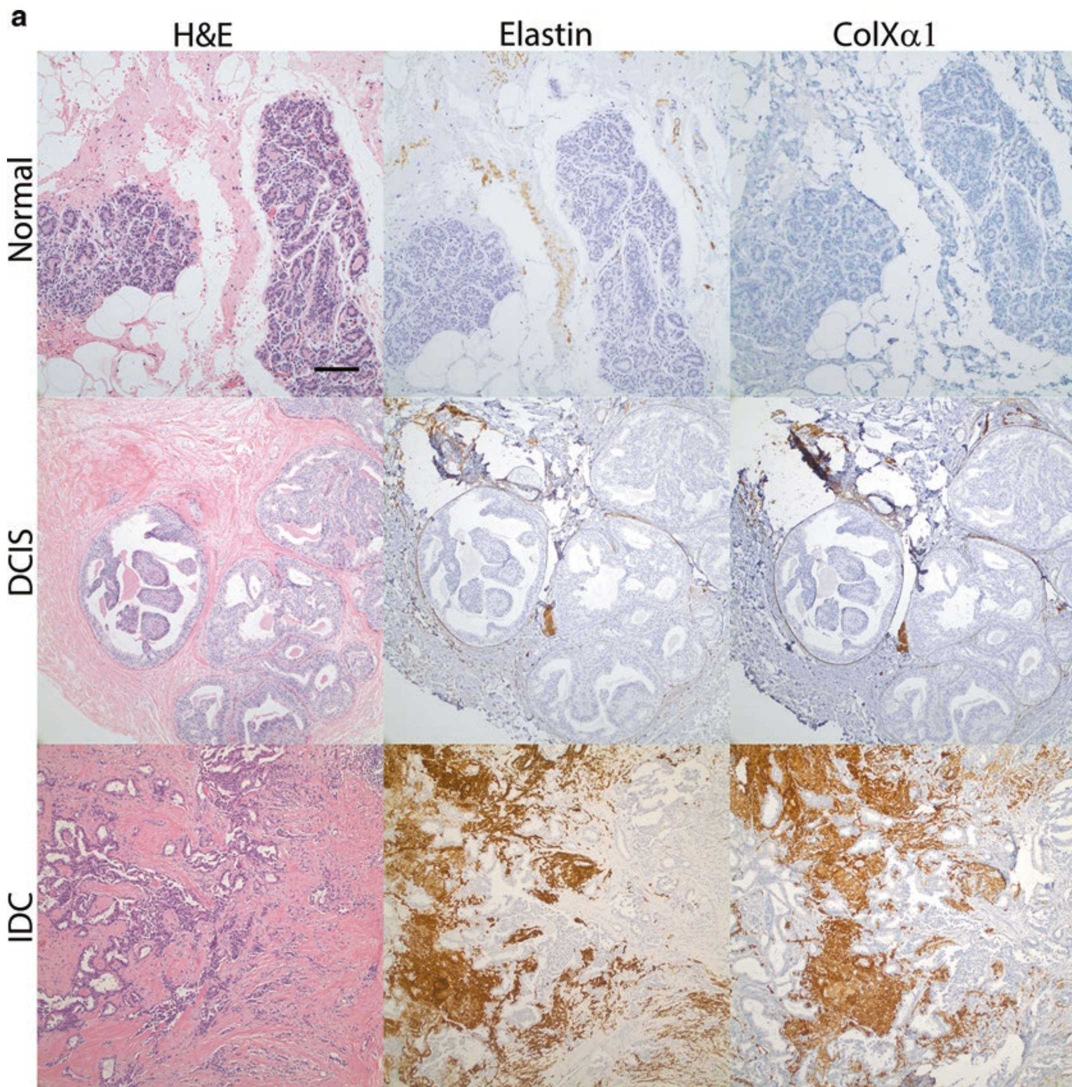

Fig. 1.4 (**a**) Elastin and collagen expression in normal breast tissue, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma. Elastin is focally expressed in normal breast stroma, but not ColXα1 (first row). In DCIS, elastin and collagen expression were both present in a periductal pattern and within the stroma (second row). In invasive carcinoma, elastin and collagen were co-expressed in tumor-associated stroma (third row). (**b**) Immunofluorescence staining of elastin and ColXα1 in tumor-associated stroma. DAPI (blue) highlights the tumor; elastin (green) and ColXα1 (red) are distributed and co-localized in tumor-associated stroma (yellow). The tumor and tumor stroma are illustrated with the merge of DAPI (tumor) and elastin (tumor stroma). (**c**) Immune electron microscopy of ColXα1 and elastin localization in breast tumor stroma using double labeling. The micrograph shows patchy amorphous material in the extracellular space near a fibroblast nucleus which is double stained with gold-conjugated anti-ColXα1 antibody (10 nm gold particles) and gold-conjugated anti-elastin antibody (25 nm gold particles). The field also contains collagen fibrils (C), collagen elastin complex (CE), and fine cytoplasmic extension of the fibroblasts (F). Original magnification (X25000). Larger particles, elastin (arrow); smaller particles, ColXα1 (in arrow head)

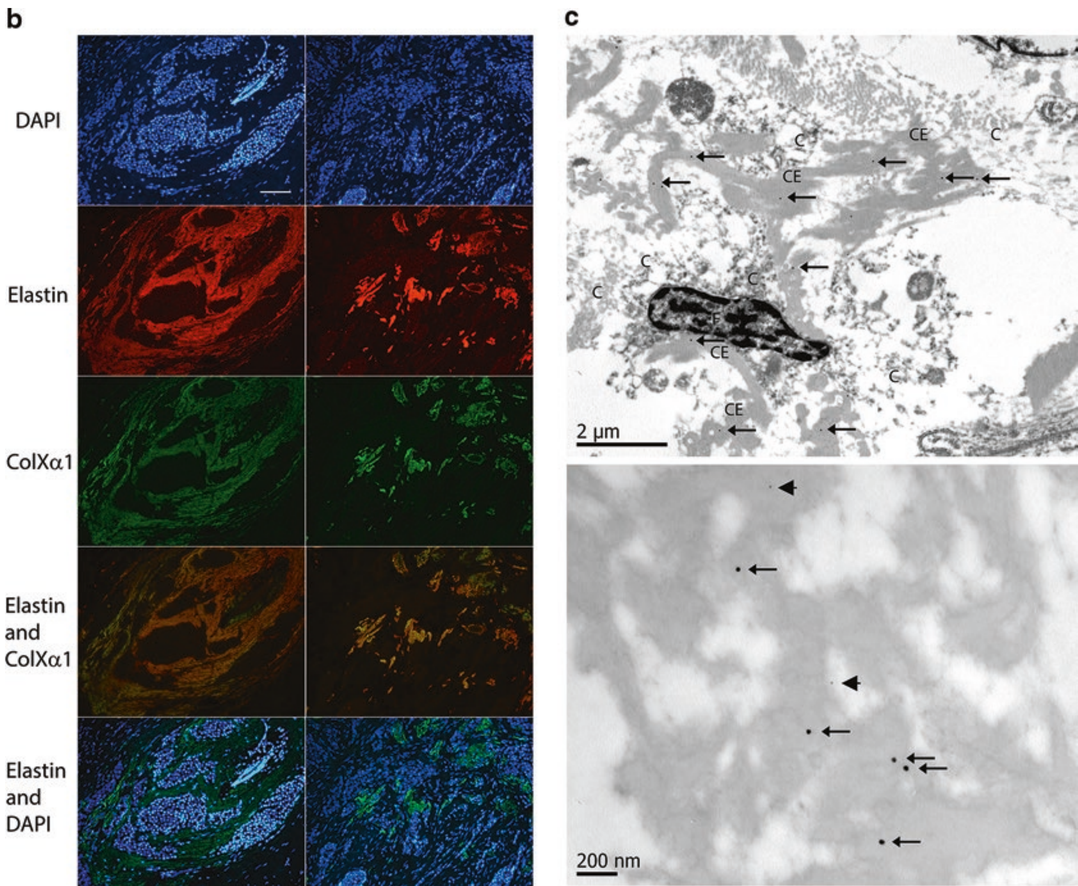

Fig. 1.4 (continued)

that ColXα1 co-localizes with elastin in invasive breast cancer-associated stroma [131] (Fig. 1.4), suggesting that this co-localization is specific for neoplastic transformation. Using radioactive labeling and HPLC methodology, Kao et al. found that the synthesis of collagen and elastin increased by 50% and 70%, respectively, in desmoplastic breast cancer stroma on a per-cell basis, further suggesting that interplay between these two proteins may be critical in the neoplastic process [132].

1.7 Future Directions

Surprisingly, despite observations dating back to more than a century, little is known of how intact elastin directly effects tumorigenesis, and even less is known of the nature of other macromolecules that interact and cross-link with elastin which also play a role in neoplasia. Newer technologies targeting the stromal-epithelial interface such as organoid culture and single cell expression analysis will likely shed light on these interactions.

Elastases, and specifically neutrophil elastase, are key drivers of invasion and metastasis and are therefore an important target for inhibition. As

neutrophil elastase is an integral component of neutrophil extracellular traps (NETs) and NETs modulate tumor growth, NETs are also potential therapeutic targets.

As stated previously in this review, due to the insoluble nature of elastin, much of the research governing elastin biology in tumorigenesis is related to EDPs and ELPs. EDPs drive tumor development in a variety of ways, and specifically targeting EDPs and their binding proteins are major objectives for ongoing and future anticancer therapies. Research on both the direct anti-cancer activity and the drug delivery capabilities of ELPs is another area likely to result in novel therapeutic agents in the near future.

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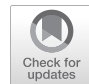

2 Decorin in the Tumor Microenvironment

Kornélia Baghy, Andrea Reszegi, Péter Tátrai, and Ilona Kovalszky

Abstract

The tumor microenvironment plays a determining role in cancer development through a plethora of interactions between the extracellular matrix and tumor cells. Decorin is a prototype member of the SLRP family found in a variety of tissues and is expressed in the stroma of various forms of cancer. Decorin has gained recognition for its essential roles in inflammation, fibrotic disorders, and cancer, and due to its antitumor properties, it has been proposed to act as a "guardian from the matrix." Initially identified as a natural inhibitor of transforming growth factor-β, soluble decorin is emerging as a pan-RTK inhibitor targeting a multitude of RTKs, including EGFR, Met, IGF-IR, VEGFR2, and PDGFR. Besides initiating signaling, decorin/RTK interaction can induce caveosomal internalization and receptor degradation. Decorin also triggers cell cycle arrest and apoptosis and evokes antimetastatic and antiangiogenic processes. In addition, as a novel regulatory mechanism, decorin was shown to induce conserved catabolic processes, such as endothelial cell autophagy and tumor cell mitophagy. Therefore, decorin is a promising candidate for combatting cancer, especially the cancer types heavily dependent on RTK signaling.

Keywords

Decorin · Extracellular matrix · Receptor tyrosine kinase · Autophagy · Mitophagy · Inflammation · SLRP · Tumor · Stroma · EGFR · Met · Angiogenesis · Cell cycle · Signaling · Growth factor

2.1 Introduction

Cancer is a rising pandemic and a major public health concern worldwide, with about 18 million new cases and over 19 million cancer deaths each year [1, 2]. Understanding the pathophysiology and molecular mechanisms of tumorigenesis is fundamental to the development of new therapeutic agents and methods against the disease. Neoplastic growth has long been viewed as a result of activating mutations in oncogenes and silencing of tumor suppressor genes in tumor

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cells that collectively provide selective advantage for proliferation, survival, and migration. However, the discovery of a myriad changes in the tumor microenvironment that is known to coordinate many aspects of tumorigenesis has shifted this paradigm. The tumor stroma is now considered as an active participant that plays determining roles by enhancing growth promoting signals, downregulating apoptotic mechanisms, and facilitating neoangiogenesis [3]. Consequently, the last decade has seen a surge of interest in the role of the stroma in carcinogenesis, and research is now focusing not only on tumor cells but also on the surrounding tumor stroma, including the abnormal synthesis and deposition of proteoglycans.

The extracellular matrix (ECM) is a complex, well-organized structure of macromolecules that interact with each other and with the resident cells of the tissue. Matrix macromolecules provide structural integrity and influence cell growth, migration, and differentiation. During the process of tumorigenesis, the ECM undergoes quantitative and qualitative changes. The tumor stroma consists of ECM components such as proteoglycans (PGs), collagens, fibronectin, laminins, hyaluronic acid (HA), (glyco)proteins, as well as growth factors, chemokines, and cytokines stored within the ECM. Beside noncellular components, various cell populations such as immune cells, fibroblasts, endothelial cells reside in the matrix, and together with tumor cells, they are responsible for ECM production [4, 5]. Tissue remodeling is a characteristic feature of tumor development, whereby changes in the number and types of resident cells occur together with the radical transformation of ECM structure and function [6]. The remodeled ECM of the tumor stroma, enriched in proteoglycans, typically supports the cancerous phenotype and promotes cancer cell aggressiveness [5, 6]. The diverse regulatory features ECM molecules can display are well exemplified by the family of small leucine-rich proteoglycans (SLRP) [7–9].

Decorin is the prototypic and best-characterized member of the SLRP family. Decorin was originally discovered as a strong binding partner of collagen necessary for proper fibrillogenesis; its appearance as "decoration" on collagen fibrils led to the eponym decorin [10–14]. The DCN gene was cloned in 1986, and the protein was initially thought to be only a structural constituent of the ECM [15]. Soon, however, it was discovered that decorin was able to affect multiple cellular functions such as proliferation, differentiation, migration, and spreading, as well as inflammatory responses [16–19]. These early studies conducted on tumor cells were the first reports on the tumor-suppressive effects of decorin and initiated the efforts, still ongoing, to leverage the anticancer properties of decorin for tumor therapy [20].

This chapter will highlight the cancer-related aspects of the cellular and molecular roles of decorin. We will first describe its distribution in cancer. Next, the structural basis of its antitumor properties will be discussed, followed by a detailed examination of the classical and novel signaling mechanisms of decorin-mediated oncosuppression. Finally, we will provide an overview of studies aiming at the therapeutic application of decorin against cancers.

2.2 The Emergence of Decorin as an Oncosuppressive Molecule

2.2.1 Misexpression and Localization of Decorin in Cancer

To understand the biological role of decorin in cancers, its expression patterns and localization within tumors need to be discussed first. Compared to the vast number of studies in vitro, the expression of decorin in tumorous tissues is relatively unexplored. Nevertheless, existing reports on decorin expression in various tumors of different grade and origin reveal a general tendency of downregulation in the parenchyma of advanced tumors, occasionally counterpointed by marked overexpression in the stroma. In human breast carcinoma, decorin is downregulated both at the mRNA and protein level compared to normal tissues as well as to non-tumorous

adjacent tissues of cancer patients [21]. Similar tendencies of decorin downregulation were observed in several other malignancies, such as lung [22], ovarian [23], and endometrial cancers [24]. In addition, in node-negative invasive breast carcinoma and soft tissue tumors, reduced expression of decorin is associated with poor prognosis [25, 26]. According to the Human Protein Atlas database, significant reduction in decorin levels was found in the stroma of several tumor types such as the breast, cervical, bladder, colon, kidney, pancreas, ovary, prostate, skin, stomach, rectal, and testis [9, 27]. Decorin expression was diminished in the stroma of low- and high-grade urothelial carcinoma, while high levels of decorin were seen in the submucosa and deep tumor stroma [28]. Decorin expression was found to be decreased in multiple myeloma and monoclonal gammopathy of undetermined significance [29], in esophageal squamous cell carcinoma [30], and in some cases of colon carcinoma [31]. Within the tumor parenchyma, several studies reported the complete absence of decorin expression in various tumors including urothelial, prostate, myeloma, and liver cancer [32–37].

While tumor cells and non-tumorous connective tissues usually contain no or little decorin, the tumorous stroma may produce large amounts of this proteoglycan as seen in human colon [38–40] and breast carcinomas [41, 42]. In these cases it is conceivable that despite its loss from tumor cells, the stromal accumulation of decorin still impedes tumor growth by forming a physical barrier as seen in desmoplastic reactions. In addition, large amounts of decorin in the ECM of stromal origin may inhibit receptor tyrosine kinases (RTKs, see details later) on tumor cell membranes in a paracrine fashion. Along with other proteoglycans, decorin expression is also highly upregulated in the ECM of pancreatic carcinoma, typically seen at the border of tumorous areas, and pancreatic stellate cells actively overproduce decorin [20, 43, 44], whereas pancreatic cancer cells exhibit a complete loss of decorin.

Intriguingly, transcriptional analysis of tumor progression at the mRNA level revealed high decorin expression during the early stages of tumorigenesis. In B-cell chronic lymphoid leukemia (CLL), high decorin mRNA levels were detected in early stages in contrast to suppression in advanced stages. In line with this, patients with nonprogressive CLL exhibited significantly higher decorin expression than those with the aggressive type [45]. Similarly, while benign hemangiomas displayed relatively high decorin mRNA levels, the transcription of decorin was completely blocked in malignant vascular sarcomas [46]. Therefore, it seems that malignant behavior and tumor progression may be correlated with the loss of endogenous decorin expression, which may serve as a biomarker for distinguishing between early- and late-stage diseases.

2.2.2 Genetic Evidence for Decorin as a Tumor Suppressor

Further evidence for the antitumor properties of decorin emerged from experimental mouse models where the gene of decorin was unconditionally knocked out [47]. Mice with ablated decorin gene developed spontaneous intestinal tumors when fed with high-fat diet [48]. In this model, loss of decorin resulted in perturbed intestinal maturation including decreased cell differentiation and increased proliferation, which were linked to the downregulation of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, intestinal trefoil factor, and E-cadherin, as well as the upregulation of β-catenin signaling [48]. The identification of these signaling molecules paved the way for further research into the antitumor mechanisms exhibited by decorin. Simultaneous genetic ablation of both decorin and p53 led to the formation of aggressive T-cell lymphomas and premature death of these animals [49]. Genetic loss of decorin also resulted in enhanced tumor incidence and tumor count in livers of mice exposed to hepatocarcinogens [36].

Collectively, these studies indicate that the loss of decorin is permissive for tumorigenesis and anticipates its tumor repressive role in cancer.

2.3 The Structure of Decorin

Structurally, decorin is highly conserved across different species. The mammalian DCN gene is located on chromosome 12q21-q22 and contains eight exons. The synthesis and secretion of decorin chiefly occur in the rough endoplasmic reticulum and the Golgi apparatus of fibroblasts, smooth muscle cells, and macrophages [50]. Decorin comprises a 42 kDa protein core with a central domain harboring 12 leucine-rich repeats (LRR) and an N-terminal attachment site for a single glycosaminoglycan (GAG) chain of chondroitin or dermatan sulfate [15, 51] (Fig. 2.1). The 12 LRRs (designated with roman numerals I–XII) form a horseshoe or banana shape [52, 53] with 14 curved β-sheets on the concave surface and α-helices in the convex region [54, 55]. Due to its unique structure, this central domain can interact with a variety of proteins and is responsible for the diverse biological functions of decorin [56]. Although the GAG chain has been shown to be important for some decorin/ligand binding, such as regulating collagen fibrillogenesis [13], most decorin-binding partners interact with decorin at its core protein. Different LRRs possess unique functional properties and contribute to specific bioactivities of decorin. LRRs V–VI constitute the binding site for vascular endothelial growth factor receptor-2 (VEGFR2) [57] and epithelial growth factor receptor (EGFR) [58]. LRR VII located on the concave surface of the core protein acts as a high-affinity binder of collagen I, the most well-known partner of decorin [59]. LRR XI is known as the "ear" repeat whose truncations or mutations may cause congenital stromal corneal dystrophy [14, 60]. Finally, LRR XII is responsible for the interaction with CCN2/CTGF [61] (Fig. 2.1a).

Albeit decorin forms homodimers in physiological solutions [54] (Fig. 2.1b), monomeric decorin appears to be the active form of the proteoglycan accounting for most of its interactions [62, 63]. Dimerization blocks the central domain, thereby preventing interactions with other substrates. However, recent studies demonstrated that decorin dimerization is reversible and the proteoglycan is able to alternate between the homodimer form and the collagen-binding monomeric form [62]. The same study reports that dimerization is not essential for the stabilization of decorin [62].

By binding to a variety of substrates via its core protein, monomeric decorin can function as a soluble paracrine factor that modulates numerous downstream signaling pathways [64].

Recently, several studies have identified decorin as a substrate of proteases. Matrix metallopro-

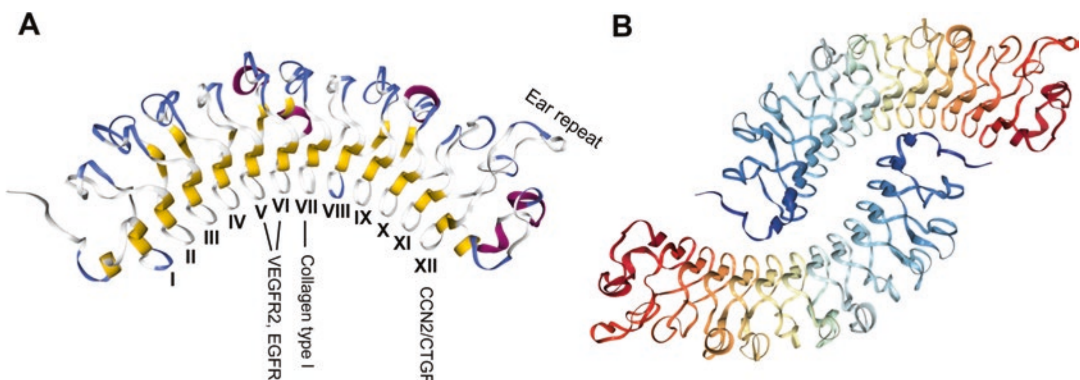

Fig. 2.1 The crystal structure of decorin. (**a**) Cartoon ribbon diagram of monomeric bovine decorin. B-strands are displayed in yellow, α-helices appear in blue. Leucine-rich repeats are designated by roman numerals. LRR V–VI represents the binding site of VEGFR2 and EGFR tyrosine kinases. LRR7 interacts with collagen type I, while LRR XII is responsible for CCN2/CTGF binding. The ear repeat plays a role in proper folding of the proteoglycan. (**b**) Dimeric crystal structure of decorin. In physiological solutions decorin exists as a dimer, a form where LRR repeats are hidden, which prevents interactions with most of its substrates. (PDB association number: 1XCD)

teinase-2 (MMP-2), MMP-3, MMP-7, and membrane type 1-matrix metalloproteinase (MT1-MMP) are all able to cleave decorin, resulting in the inactivation of the molecule [65]. Likewise, BMP-1 peptidase also processes decorin in a similar manner [66]. In addition, decorin can also be inactivated by proteases secreted by inflammatory cells. Decorin as a member of damage associated molecular patterns (DAMPs) may be recognized by pattern recognition receptors such as Toll-like receptor 2 (TLR2) and TLR4 inducing an inflammatory response [67].

2.4 Roles of Decorin in Inflammation and Immunomodulation

In experiments of tissue stress and injury, it has become evident that decorin, as well as its relative molecule biglycan, can regulate the innate immune response and inflammatory responses via TLR2 and TLR4 [56, 67–70] (Figs. 2.2 and 2.3). Decorin is able to modulate inflammation through a number of mechanisms. It can directly engage TLR2 and TLR4 on the surface of macrophages leading to transient activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways (Fig. 2.3). These events enhance the secretion of inflammatory factors such as tumor necrosis factor-α (TNFα) and IL-12p70 [71]. Furthermore, interaction with TLR2 and TLR4 can stimulate the production of the proinflammatory programmed cell death protein 4 (PDCD4) by macrophages [68] (Fig. 2.3). Indirectly, by downregulating the bioactivity of transforming growth factor-β1 (TGF-β1), decorin can counteract the transcriptional repression of PDCD4 via inhibition of microRNA-21 (miR-21) [68]. As a consequence, anti-inflammatory mediators such as IL-10 are translationally suppressed by PDCD4, which creates a proinflammatory tumor microenvironment ([50, 56, 64, 68, 72] (Fig. 2.3). The ability of decorin to regulate inflammation is important for understanding its role in tumor biology, as a proinflammatory tumor microenvironment retards tumor growth [7, 50, 64, 73]. In addition, by stimulating CCL2 production, decorin recruits mononuclear cells to the site of injury that results in a sustained inflammatory state [74]. Its interaction with the Class A scavenger receptors expressed on the surface of macrophages promotes the adhesion of these cells to the matrix [75].

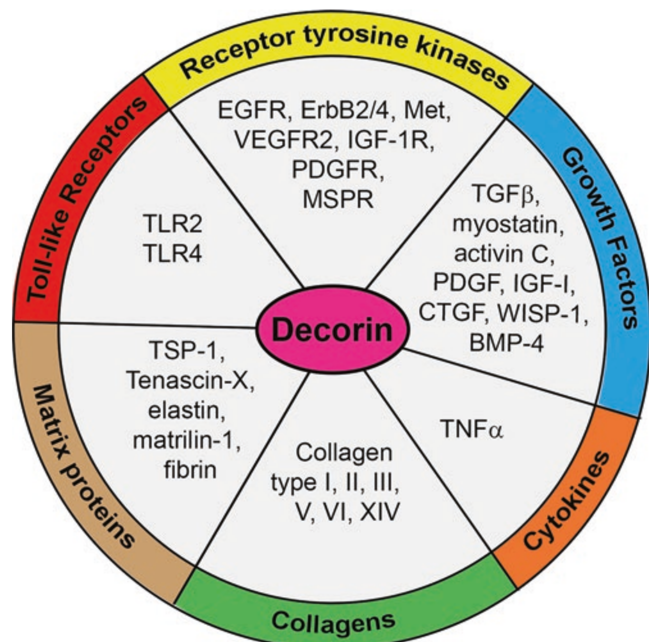

Fig. 2.2 Interacting partners of decorin. Growth factors, receptors, and ECM molecules that are regulated by decorin via physical binding with high affinity. Further details are provided in the text

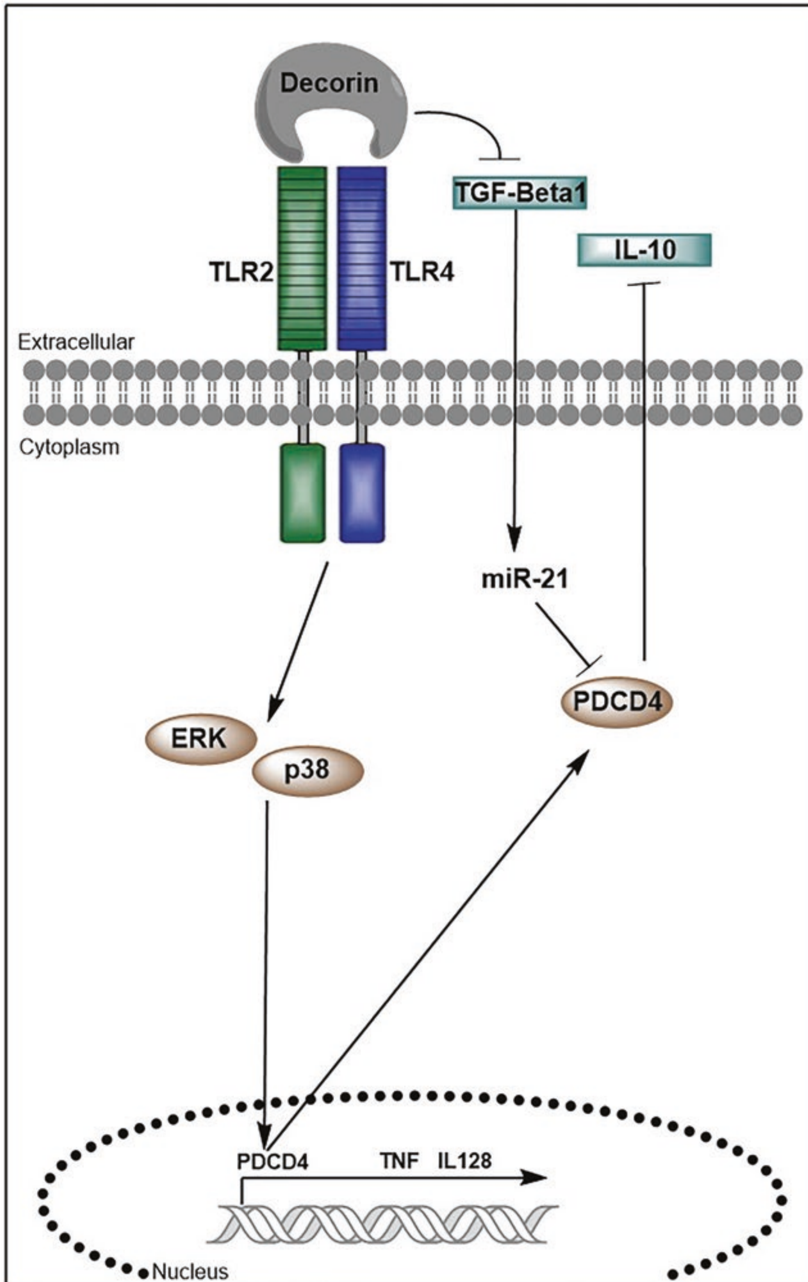

Fig. 2.3 Immunomodulatory actions of decorin. Decorin engages proangiogenic signaling pathway via activation of TLR2 and TLR4 in concomitant with TGFβ inhibition. As a result, PDCD4 is transcriptionally activated via inhibition of miR-21 and suppresses the anti-inflammatory protein IL-10

In addition to the effects of decorin on macrophages, decorin can modulate the behavior of leukocytes. In a mouse model of delayed-type hypersensitivity (DTH), Seidler and coworkers reported that decorin mediates DTH responses by influencing polymorphonuclear leukocyte attachment to the endothelium [76]. This occurs via two mutually nonexclusive mechanisms, a direct anti-adhesive effect on polymorphonuclear leu-

kocytes and a negative regulation of ICAM-1 and syndecan-1 expression [76].

Despite the ability of decorin to induce proinflammatory responses, decorin core protein was also reported to downregulate chemotactic and inflammatory genes in leukocytes [77]. Thus, it seems that the roles of decorin in regulating inflammation and immune reactions are complex, and further studies are necessary to clarify whether decorin in each specific context initiates pro- or anti-inflammatory responses. Merline and coworkers reported that intact decorin, but not the protein core or GAG chain alone, was able to increase TNFα and IL-12p70 production [68]. Buraschi et al., on the other hand, observed inhibition of anti-inflammatory gene transcription when applying the protein core only [77]. Therefore, it is conceivable that the holomeric molecule and the core protein act oppositely, with the latter competitively binding to TLR receptors and repressing inflammation in the tumor stroma [50]. Decorin knockout animals display proinflammatory phenotype in different in vivo models of fibrotic diseases, while addition of exogenous decorin suppresses inflammation in experimental treatment trials [18, 78–81]. Tumor necrosis factor-α (TNF-α), a major inflammatory cytokine, is a known binding partner of decorin [82]. Upon their interaction, the cytokine is sequestered and prevented from exerting its proinflammatory effects on its receptors.

2.5 Decorin as a Multifaceted Tumor Suppressor: Signaling in Cancer

2.5.1 Interaction with Growth Factors

The first growth factor discovered to interact with decorin was transforming growth factor-β (TGF-β) [83] (Fig. 2.2). Binding of TGF-β by decorin effectively inhibits proliferation of tumor cell lines dependent on this growth factor and the spread of cancer [19, 83]. The protein core of decorin recognizes and binds to all isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) [84]. To explain the mechanism of antitumor action, it has been proposed that collagen-bound decorin sequesters TGF-β and anchors it to the ECM, thereby preventing its interaction with TGF-β receptors on the cell surface [80]. Indeed, decorin exposure inhibits TGF-β signaling in vitro [64, 85, 86] and indirectly attenuates downstream signaling pathways as reported in a mouse liver fibrosis model [87]. By binding to TGF-β and forming an inactive complex, decorin blocks signaling via Smad2, Smad3, and Erk1/2 and thus curbs fibrogenesis [88]. TGF-β, in turn, inhibits decorin mRNA transcription in fibroblasts [89], which suggests a feedback loop responsible for maintaining the homeostasis of matrix deposition. TGF-β is an important cytokine in the regulation of inflammation, and decorin as a physiological TGF-β-inhibitor limits the duration of TGF-β responses in inflammation and tissue repair [18, 19, 80]. In cancer, the role of TGF-β is contradictory as it can either suppress or promote tumorigenesis, and its mode of action highly depends on the cellular context [90]. Thus, the neutralization of TGF-β by decorin as a potential anticancer strategy needs careful evaluation.

Decorin also binds to and inhibits the action of myostatin, another member of the TGF-β superfamily [91, 92] (Fig. 2.2). In this case, decorin sequesters myostatin and attenuates its growth inhibitory effects on myofibers, which results in improved muscle regeneration [91–93]. Myostatin has also been recognized as an important player in the development and maintenance of cancer cachexia [94–96]. Myostatin antagonists emerge as promising novel therapeutics against cancer cachexia, as they not only prevent muscle wasting but may also have a beneficial effect on the overall survival [94–96]. Thus, by quenching the action of myostatin, decorin may be able to antagonize cancer-related wasting.

Via its LRR XII repeat decorin interacts with and negatively regulates connective tissue growth factor (CTGF) [61, 64] (Fig. 2.2). CTGF plays important roles in the progression of fibrosis [97], as well as in the regulation of ECM production, chemotaxis, cell proliferation, and differentiation, and also modulates inflammation [98, 99]. CTGF is tightly regulated by TGF-β and

stimulates decorin expression, suggesting a strict mechanism of autoregulation in this pathway [61].

Wnt-inducible signaling pathway protein-1 (WISP-1, alias CCN4) belongs to the CTGF family and has been identified as an oncogene in a number of cancers, where it enhances cell migration and promotes epithelial-to-mesenchymal transition [100]. Decorin was shown to bind WISP via its dermatan sulfate GAG chain and may thus act as a regulator of Wnt signaling [72, 101] (Fig. 2.2). Decorin is known to downregulate β-catenin [48, 102]; however, a direct connection between decorin binding to WISP-1 and inhibition of β-catenin has not been demonstrated.

The oncogenic activin C, another relative of TGF-β has also been recognized as a binding partner of decorin (Fig. 2.2). Their interaction induces caveolin-mediated endocytosis and degradation of the growth factor leading to inhibited proliferation and migration of colorectal cancer cells [103].

Another example of inhibition by direct sequestration is the interaction of decorin with platelet-derived growth factor (PDGF) (Fig. 2.2). In this way, decorin prevents PDGF-dependent phosphorylation of its receptor that results in attenuated signaling in liver cancer [36, 104] and prevents cellular migration in intimal hyperplasia [105].

Decorin plays an important role in the regulation of the insulin signaling pathway. Beside its interactions with IGF-1R and insulin receptors (see later), decorin also binds to IGF-I with low affinity and competes for the growth factor with its endogenous binding partners [64, 106] (Fig. 2.2). In this scenario, the action of decorin is highly concentration dependent, and with increased amounts (e.g., in a therapeutic context), it could effectively attenuate signaling by IGF-I [64].

Although not belonging to the family of growth factors, it is important to note that decorin also binds to a variety of structural components within the ECM, such as different types of collagens, tenascin X [107], and elastin [108] (Fig. 2.2). Importantly, decorin binds to collagen VI with high affinity and with matrilin-1 it directly links collagen VI to aggrecan or collagen type II fibrils [109]. These observations highlight the importance of decorin in regulating matrix structure formation and desmoplastic reactions in the tumor stroma [72]. The active contribution of decorin to stromal reaction, a defensive mechanism of the host tissue against cancer, represents another skill in its tumor suppressor repertoire.

2.5.2 Decorin Acts as a Pan-RTK Inhibitor

The most well-known tumor inhibitory action of decorin is its ability to interact with and directly engage a multitude of receptor tyrosine kinases (RTKs) (Fig. 2.2). This pan-inhibition of RTK pathways earned decorin the reputation of "the guardian from the matrix" [72].

The first RTK discovered to interact with decorin was epidermal growth factor receptor (EGFR), a member of the ErbB receptor family. Detailed analysis in the A431 squamous carcinoma cell line revealed that monomeric decorin binds to a narrow region of the receptor partially overlapping with the binding site of EGF [58, 110–112]. This interaction evokes receptor dimerization and transient autophosphorylation of EGFR leading to caveolin-1-mediated internalization and lysosomal degradation of the receptor [72, 113]. In contrast with phosphorylation induced by the endogenous ligand of EGFR, this interaction elicits cell cycle arrest, apoptosis, angiostasis, and protracted oncogene suppression [9, 64, 72]. The decorin-initiated phosphorylation of EGFR activates the MAPK signaling cascade and subsequent intracellular Ca^{2+} release [114, 115] and induces the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and cleavage of caspase-3 [114] (Fig. 2.4). Other studies have also reported inhibition of EGFR and its downstream signaling by decorin. For example, in A549 lung carcinoma cells, overexpression of decorin decreased EGFR activity, which induced G1 phase block and apoptosis of the tumor cells via elevated p53 and $p21^{WAF1/CIP1}$ expression [116]. Conversely, the lack of decorin resulted in

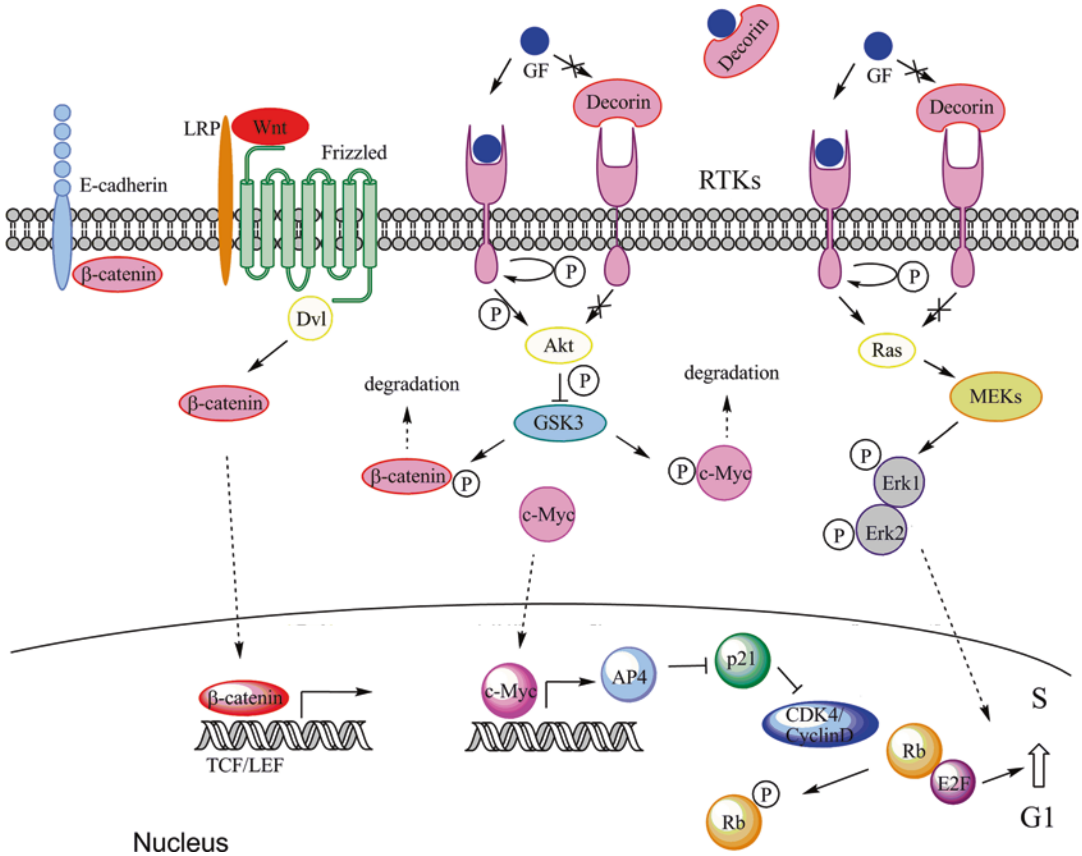

Fig. 2.4 Emerging signaling pathways affected by decorin. Antagonism of a multitude of receptors by decorin results in attenuated downstream signaling cascades culminating in antiproliferative and antiangiogenic mechanisms in tumor cells. A detailed description is provided in the text

enhanced EGFR phosphorylation followed by activation of ERK1/2 in experimental models of liver carcinogenesis [36] (Fig. 2.4).

Other members of the ErbB family, especially ErbB2 and ErbB4, also interact with decorin [64, 117, 118]. By binding to ErbB4 decorin prevents dimerization of ErbB4 with ErbB2, which arrests growth and prompts differentiation in breast carcinoma cells [117].

Another significant interacting RTK partner of decorin is the Met receptor (hepatocyte growth factor receptor, scatter receptor) [119]. Upon binding to decorin, Met undergoes strong Tyr phosphorylation. Similar to the mechanism seen in the case of EGFR, this provokes the recruitment of c-Cbl and, ultimately, proteasomal degradation of the receptor [9, 119]. Attenuation of Met by decorin suppresses downstream signaling molecules Myc and β-catenin and thereby inhibits tumor growth [119, 120] (Fig. 2.4). In addition, attenuated activity of Met by decorin induces the antiangiogenic protein TIMP-3 with a concurrent decrease of the proangiogenic proteins HIF-1α and VEGFA [121].

In recent years, many other RTKs were discovered to mediate the bioactivities of decorin. These include IGF-1R, insulin receptor (IR), and their ligands [104, 106, 122, 123]; PDGFRα and its ligand PDGF [36, 104]; VEGFR2 [57, 124]; and MSPR (RON) [36] (Fig. 2.2). As opposed to the general scheme of caveosomal endocytosis and degradation of RTKs upon decorin binding, IGF-1R is not internalized and tagged for destruction. In this case, decorin attenuates signaling of IGF-1R through IRS-1, IGF-1, and Akt/ERK/p70S6K, resulting in a migratory block. In addi-

tion, decorin prevents IGF-1 from localizing IGF-1R to caveosomes [28, 72, 123, 125]. It was suggested, however, that decorin inhibits IGF-1R only in cancers but acts as an IGF-1R agonist in normal tissues [56, 106]. A recent study comparing signaling pathways evoked by decorin in four different hepatoma cell lines reported that IGF-1R as well as IR can be either enhanced or inhibited even in cancer cell lines with the same tissue origin [126]. Also, within the same cell line, initial phosphorylation of IGF-1R and IR upon decorin treatment decayed rapidly, and inhibited receptor activity was observed after 2 days. These findings underline that the effect of decorin on IGF-1R regulation is complex and may be affected by unique features (e.g., differentiation state) of the particular tumor [126].

In summary, the interactions between decorin and various RTKs elicit numerous changes in cell signaling pathways with the cumulative effect of attenuated tumorigenesis [64]. Since many solid tumors depend upon RTK signaling, they may be profoundly inhibited by the introduction of decorin [9, 34, 36, 117, 127].

2.5.3 Intracellular Signaling Pathways Activated by Decorin

Consequent to the inhibition of receptors, decorin efficiently attenuates downstream signaling pathways involved in tumor cell proliferation, survival, and angiogenesis. Decorin-mediated antagonism of the Met receptor leads to selective degradation of β-catenin and Myc oncoproteins [9, 120] (Fig. 2.4). Binding of hepatocyte growth factor (HGF), the endogenous ligand to Met, initiates a signaling cascade resulting in the stabilization of β-catenin by direct phosphorylation and by simultaneous repression of the function of glycogen synthase kinase-3β (GSK-3β) via phosphorylation [9, 120, 121] (Fig. 2.4). The stabilization of β-catenin seems to occur independently from Wnt signaling and triggers nuclear translocation of β-catenin and transcriptional activation of its target genes such as the oncoprotein Myc driving pro-tumorigenic and pro-survival signals [3, 120]. Myc is a transcription factor that coordinates a wide variety of molecules stimulating cell proliferation. One of the targets of Myc is AP4, a transcriptional repressor of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ [128] (Fig. 2.4). Cell cycle suppression through the induction of p21$^{WAF1/CIP1}$ by decorin was reported in many cancer models and partly accounts for decorin's ability to attenuate tumorigenesis [72, 129]. As a consequence of Met inhibition by decorin, both β-catenin and Myc are targeted for degradation via the 26S proteasome [56, 120] (Fig. 2.4). As a result, β-catenin fails to translocate to the nucleus and remains in the cell membrane upon decorin exposure [126] (Fig. 2.5). The transcriptional repression and phosphorylation-dependent degradation of Myc (at Thr58 residue) induced by decorin leads to transcriptional activation of *CDKN1A* locus via loss of AP4 repressor [36, 120] (Fig. 2.4). The increase in phosphorylated Myc and β-catenin may be a result of derepressed GSK-3β downstream of attenuated Met signaling. Although the aforementioned signaling pathway was first described in the case of Met receptor, the same may apply for other RTKs. Downstream of RTKs, the Ras/MEK/ERK, and PI3K/Akt/mTOR the major and best-studied pathways in several types of cancers [130, 131]. The active form of Akt is known to inactivate GSK-3β via phosphorylation [132, 133], which is a key molecule linking several signaling pathways such as those originating from both Wnt and RTKs. Indeed, in an experimental model of hepatocarcinogenesis, ablation of decorin gene resulted in activation of multiple RTKs with enhanced MAPK and Akt pathways, in parallel with decreased degradative phosphorylation of both β-catenin and Myc [36] (Fig. 2.4).

Within the nucleus, c-Myc induces AP4 expression that, in turn, represses p21$^{WAF1/CIP1}$. Reduced p21$^{WAF1/CIP1}$ levels are insufficient to inactivate CDK4/CyclinD1. In this way, Rb phosphorylation culminates in E2F release, thereby allowing the cell to bypass the restriction point in G1 phase [36] (Fig. 2.4). In addition to p21$^{WAF1/CIP1}$, decorin was shown to upregulate other cyclin-dependent kinase inhibitors such as p27^{KIP1} [48, 134, 135], p15^{INK4b} [136], and p57^{KIP2}

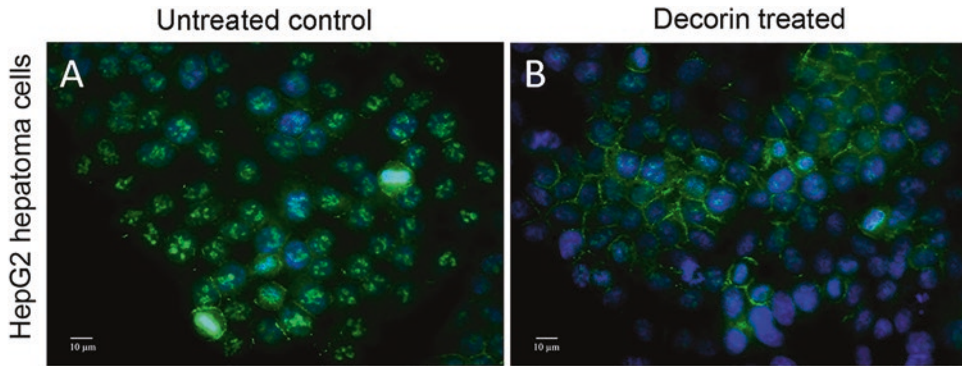

Fig. 2.5 Decorin exposure retains β-catenin in the plasma membrane, preventing its signaling within the nucleus. Detection of β-catenin in HepG2 liver cancer cells by immunofluorescence

[137], preventing cell cycle progression from G1 to S phase. Attenuation of β-catenin-driven cyclin D1 expression adds another important component to the cell cycle blockade [72, 120]. While earlier reports all demonstrated that decorin induces cell cycle arrest at the G1/S transition, a recent comparative study revealed that it is also able to induce G2/M arrest in hepatoma cells [126] (Fig. 2.6). The Hep3B hepatoma cell line used in this study harbors deleterious mutations of both p53 and retinoblastoma (Rb) genes [138]; therefore, cell cycle arrest at the G1/S restriction point is compromised, and cells will unconditionally cross the checkpoint. It is a known phenomenon that increased Akt activity may lead to hyper-replication ending up in replication stress that activates the ATR/Chk1/Wee1 system stopping the cell cycle at the G2/M transition via phosphorylation of CDK1 [139]. Indeed, in Hep3B cells, the levels of pCDK1 and Wee1 increased in parallel with the high phospho-Akt levels which, in turn, originated from activated IR and IGF-1R; simultaneously, the expression of Cdc25A phosphatase decreased, supporting the mechanism proposed [126]. These experiments demonstrated that decorin not only inhibits the G1/S phase transition but is also capable of blocking cell cycle progression at a later stage at the G2/M checkpoint (Fig. 2.6) [126]. This inhibitory effect of decorin had been unknown prior to this publication, and while more studies are needed to reveal the underlying signaling processes, the newly discovered impact of decorin on the G2/M checkpoint further substantiates the tumor suppressor ability of decorin. As the p53 and Rb tumor suppressors are among the most frequently mutated genes in cancer, the fact that decorin is able to exert its antiproliferative action even in the absence of functional p53 and Rb adds another argument in favor of its application against cancer. Of note, the very same study proved that decorin evokes completely different cellular responses and signaling pathways in different tumor cell lines, all derived from hepatocellular carcinoma [126]. Therefore, it must be kept in mind that the impact of decorin is highly cell type-specific.

2.5.4 Decorin Induces Endothelial Cell Autophagy and Tumor Cell Mitophagy

The list of decorin's antitumor activities was further expanded with the discovery that decorin is able to indirectly induce vascular endothelial cell autophagy resulting in inhibited spread and metastasis of tumor cells (Fig. 2.7). Mechanistically, decorin evokes a prolonged autophagic program via transcriptional induction of Peg3 (paternally expressed 3) tumor suppressor [50, 56, 77]. PEG3, an imprinted gene, is epigenetically silenced via promoter hypermethylation of the active allele in multiple gynecologic and neural tumors [140–143]. Furthermore, Peg3 noncanonically suppresses

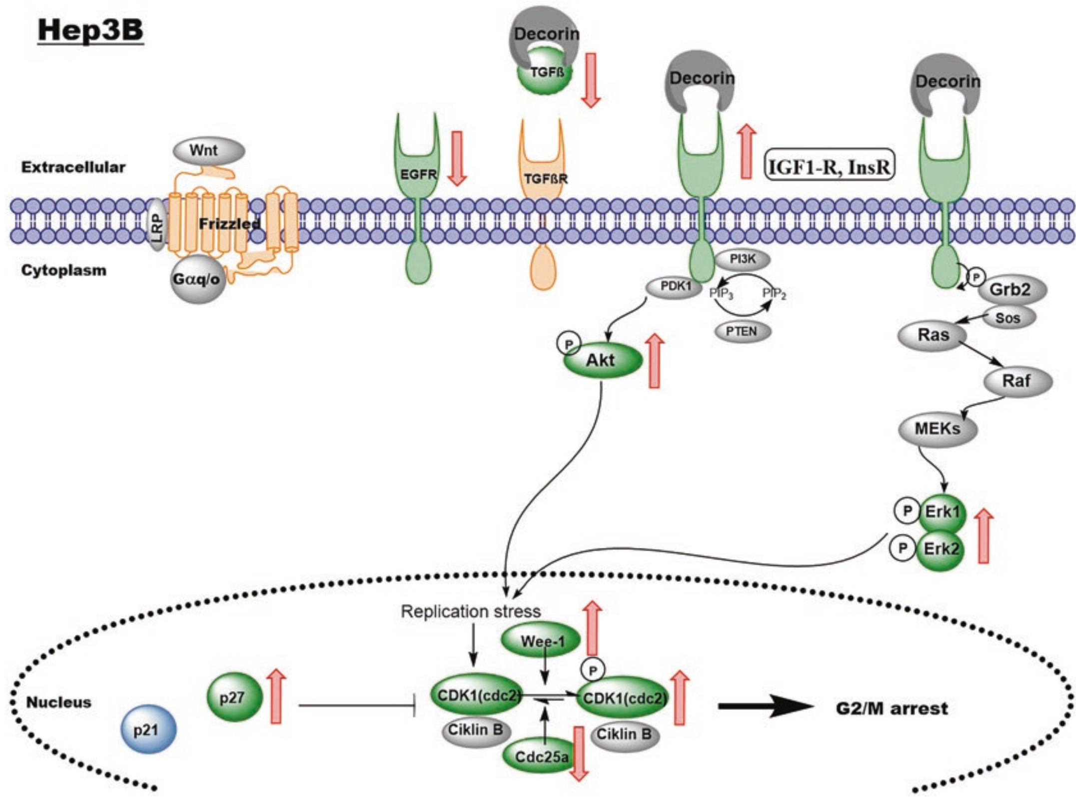

Fig. 2.6 Cell cycle arrest at the G2/M phase provoked by decorin. In the p53 and retinoblastoma double-mutant hepatoma cell line Hep3B, where G1/S arrest is compromised, activation of intracellular signaling pathways (Ras/MAPK and Akt) resulted in enhanced Wee1 expression with concomitant block of Cdc25a. The events ultimately led to inactivating phosphorylation of CDK1 and cell cycle blockade at G2/M

the Wnt/β-catenin signaling pathway, partly accounting for the activity of decorin in tumors [9, 144]. In tumors, autophagy acts as an inhibitor of tumor initiation by assisting the clearance of misfolded proteins, reactive oxygen species (ROS), and other factors [50, 145]. After stimulation of autophagy by starvation or mTOR inhibition, decorin binds to VEGFR2 on the cell surface of endothelial cells (Fig. 2.7). Here, in contrast to the pan-inhibition of RTKs in tumor cells, decorin acts as a partial VEGFR2 agonist for initiation of autophagy [9, 146, 147]. The decorin-receptor interaction activates the proautophagic AMPKa/Vps34 signaling pathway and concurrently inhibits the anti-autophagic PI3K/Akt/mTOR/p70S6K pathway [148, 149]. Importantly, Peg3 is required for decorin-induced transcriptional activation and accumulation of beclin-1 and the microtubule-associated protein light chain 3 (LC3) and is responsible for maintaining the basal beclin-1 level in endothelial cells [9, 146]. Collectively, these signaling events culminate in the formation of autophagy precursor complexes including Peg3, beclin-1, and LC3. In parallel, decorin attenuates the formation of the inhibitory Bcl-2/beclin-1 complex [150] (Fig. 2.7). Of note, EGFR and Akt signaling was reported to inactivate beclin-1 via phosphorylation resulting in autophagy suppression and chemoresistance [151, 152]. Since many RTKs share the same core signaling network, it is plausible that the disengagement of beclin-1 by decorin represents a general process. In addition to initiation of autophagy, decorin also compromises capillary formation [121, 147, 153]. In conclusion, decorin induces autophagy of endothelial cells, reduces the growth of blood

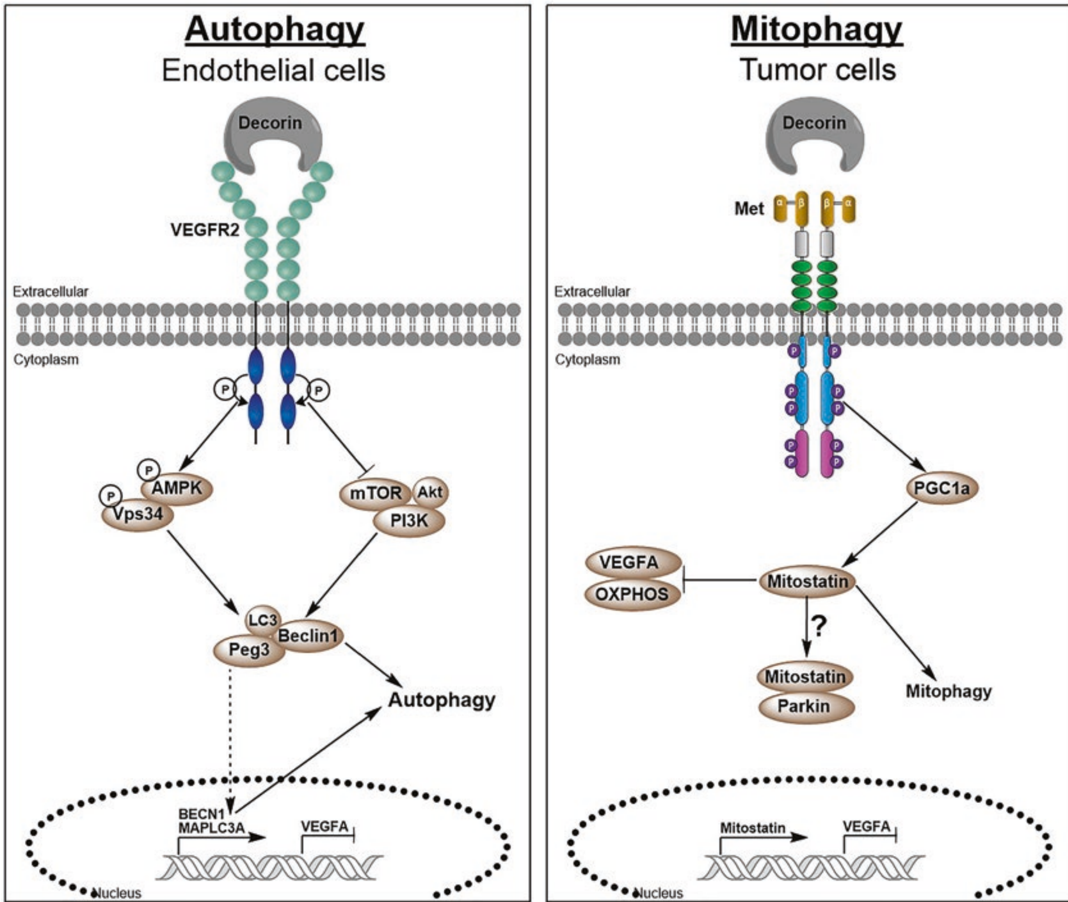

Fig. 2.7 Schematic illustrations of signaling events in decorin-mediated endothelial cell autophagy (left panel) and tumor cell mitophagy (right panel). For detailed description please refer to the text

vessels in the tumorous stroma, and is able to prevent metastasis and spread of tumor cells.

A novel mechanism of action for decorin in sustaining angiogenesis and curbing tumorigenesis has recently been unfolded, as decorin was shown to have a direct impact on catabolic processes and organelle turnover within the tumor proper [56]. Decorin was reported to induce mitochondrial autophagy (mitophagy) via induction of the Met receptor in a breast carcinoma cell line [154] (Fig. 2.7). Soluble decorin protein core effectively inhibited mitochondrial respiratory complexes and mitochondrial DNA (mtDNA) and initiated a dynamic interplay between peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and the decorin-induced tumor suppressor gene, mitostatin [154]. The interaction stabilized mitostatin mRNA and led to the accumulation of the protein, and the processes were guided by the Met/decorin axis. Blockade of mitophagic induction by depletion of mitostatin compromised decorin's ability to suppress vascular endothelial growth factor A (VEGFA) production (Fig. 2.7). Decorin also enhances mitochondrial depolarization probably via elevated Ca^{2+} levels, a consequence of the decorin/EGFR interaction [155]. As mitostatin is located at the mitochondrial associated membrane, mitostatin induced by decorin may trigger Ca^{2+} efflux from the endoplasmic reticulum into the mitochondria [56].

In conclusion, through modulation of RTK signaling decorin stimulates mitophagy, a conserved catabolic process in tumor cells and in

parallel induces endothelial cell autophagy. These newly elucidated activities of decorin offer additional keys for the control of tumor growth and neovascularization.

2.5.5 Further Roles of Decorin in Angiogenesis

Neovascularization is a fundamental step in cancer progression, whereby the growth of new vessels from preexisting blood vessels supplies the growing tumor mass. Decorin has been implicated in the regulation of angiogenesis; its role, however, remains controversial. The involvement of decorin in neovascularization was discovered during investigations of corneal development, where the contribution of decorin to angiogenesis was ambivalent [9, 156, 157]. In a normal, nontumorigenic environment, decorin supports angiogenesis by promoting integrin-collagen interaction, thus facilitating endothelial cell adhesion and migration to type I collagen and α2β1 integrin [158]. Ablation of decorin gene, in turn, leads to impaired angiogenesis in the injured cornea [156], and a decorin mimic compound promoted proliferation and migration of endothelial cells [19, 159]. Furthermore, decorin acts as an angiocrine factor (endothelial cell-derived growth factor for organ-specific tissue regeneration) for liver regeneration after partial hepatectomy [160]. Similarly, decorin protected endothelia from hyperglycemia and supported angiogenesis via IGF-1R/Akt/AP-1/VEGF signaling, which delineates a new therapeutic strategy for patients with diabetic cardiomyopathy [161]. Again, the above experiments reporting proangiogenic activity of decorin were all conducted in normal or non-tumorous models. In contrast, the greater part of literature on the role of decorin in angiogenesis focuses on tumors and underlines its antiangiogenic role [9, 72]. Decorin was reported to hinder tumor angiogenesis in a variety of tumor cell lines [153], and its expression is inversely correlated with the extent of tumor vascularization [46]. In addition, decorin induces the synthesis of matrix metalloproteinase-2 (MMP-2), which directly degrades collagen IV in the endothelial basement membrane resulting in reduced proliferation of blood vessels [50, 162]. Among the interactions of decorin with RTKs, inhibition of VEGFR2 is the most significant in the attenuation of tumor angiogenesis. Decorin binds to VEGFR2 with high affinity at a site partially overlapping with that of VEGF-A [19, 57]. Another mechanism whereby decorin exerts its antiangiogenic effect is via abrogation of the HGF/Met signaling pathway which culminates in hindered VEGF-mediated angiogenesis [121, 163]. These events lead to the repression of hypoxia-inducible factor-1α (HIF-1α), β-catenin, Myc, and Sp1 (see previous sections). Decorin also stimulates HIF-1α protein degradation [121]. Sp1 requires phosphorylation by the p42/44 mitogen-activated protein kinases (MAPK, alias ERK1/2) for activation of VEGFA transcription, which pathway may be suppressed by antagonizing RTKs [120, 121]. Within the ECM, decorin prevents the release of matrix-bound VEGFA via decreasing the expression and activity of MMP-2 and MMP-9 enzymes, which process depends on β-catenin [9, 72, 120, 121]. In parallel with the inhibition of these proangiogenic factors, decorin induces antiangiogenic molecules such as thrombospondin-1 (TSP-1) and tissue inhibitor of metalloproteinase-3 (TIMP3) [121, 164]. Decorin facilitates secretion of TSP-1 by interfering with RhoA/ROCK1 signaling [164], a cascade of many pathways including those of RTKs. In addition to influencing the balance of anti- and proangiogenic factors, decorin also utilizes the mechanism of autophagy for its antiangiogenic actions (see previous section). Decorin seems to interfere with early events of vascularization by repressing the angioplasticity of the stroma and by suppressing proangiogenic factors within the tumor parenchyma [9].

2.6 Therapeutic Approaches

In the last decade, a vast number of in vivo studies have shown that decorin administration can inhibit tumor growth and progression. Some earlier studies were built around the basic idea of tumor gene therapy, which involves incorpora-

tion of an anticancer agent into an oncolytic adenovirus [50, 165]. Genetically modified viruses that target and destroy tumor cells without toxicity to normal cells have emerged as a promising strategy [166]. Delivery of decorin via an adenovirus vector into the tumor cells inhibited the growth of lung, colon, and squamous cell carcinomas [167] by attenuating EGFR phosphorylation. Adenovirus-mediated decorin transfer inhibited Met and Wnt/β-catenin signaling pathways and thus prevented the formation of bone metastasis of prostate cancer cells [168]. Forced expression of decorin in osteosarcoma cells resulted in decreased motility and invasion of tumor cells and improved survival of animals [169]. Virus-delivered decorin attenuated breast cancer growth and prevented its metastasis formation in various organs [170–172]. Ma and coworkers found that virus-mediated decorin gene therapy prolonged survival and inhibited tumor growth in an in vivo glioma model. The rate of inhibition directly correlated with the expression levels of decorin and with the timing of DCN gene transfer [173]. Decorin gene therapy was successfully applied in models of prostate and pancreatic cancers as well [174, 175].

Decorin as a therapeutic gene against cancer may in fact hit multiple birds with one stone. Firstly, the ECM normally impedes the spread of viruses, including therapeutic viral vectors, in the tumor [50, 176]. Secondly, tumors of patients participating in clinical trials are typically characterized by a highly immunosuppressive tumor microenvironment [50, 177]. Decorin as a key organizer of the ECM has the potential to remodel the tumor stroma to enhance viral penetration, and, at the same time, it may also counter intratumoral immunosuppression by antagonizing TGF-β. Indeed, intratumoral injection of Ad-DCN, where a mutant decorin gene with increased binding affinity to collagen was applied showed greatly enhanced tumor penetration and led to improved tumor reduction and survival benefit [178]. In these experiments, increased cancer cell cytotoxicity was achieved through the action of decorin on the ECM. The same vector injected into the primary tumor greatly reduced the lung metastases formation of melanoma [178]. In a recent study, decorin was applied as an adjuvant to conquer TGF-β-mediated immunosuppression of tumors. To this end, a novel oncolytic adenovirus coding for IL-12 (a potent antitumor cytokine) and decorin was created [179]. Treated tumors showed significantly higher levels of interferon (IFN)-gamma, tumor necrosis factor-alpha, monocyte chemoattractant protein-1, and IFN-γ-secreting immune cells. Also, the vector attenuated intratumoral TGF-β expression, promoted infiltration of CD8+ T cells, and enhanced viral spread within the tumor [179]. The combined utilization of decorin with a cytokine to overcome tumor-induced immunosuppression may be a promising future avenue of cancer immunotherapy.

In addition to in vivo gene therapy studies, several successful in vitro and in vivo experiments utilizing recombinant decorin have been reported. Administration of decorin core protein to A431 squamous carcinoma cells and transfection of DCN cDNA into breast carcinoma cells inhibited EGFR activity, induced apoptosis, and hindered tumor growth [112, 115, 118]. Systemic administration of decorin inhibited the growth and metabolism of breast cancer cells and interfered with their metastatic spread to the lungs [114, 117]. Decorin was shown to synergize with carboplatin in inhibiting the proliferation of ovarian tumor cells [180] but attenuated the cytostatic effect of carboplatin and gemcitabine on pancreatic cancer cells [44]. In both models, as expected, decorin exerted an antiproliferative effect on tumor cells. These studies highlight the tissue specificity of decorin's action and remind that in clinical settings, the ability of decorin to modulate the efficiency of chemotherapeutics must be taken into account.

Despite its lack of toxicity and the treatment success seen in a large number of preclinical studies as well as in in vivo anticancer and antifibrotic experiments, decorin has not yet been developed into a clinical drug. A major reason for that is that decorin as a proteoglycan is difficult to mass produce, since due the heterogeneity of its GAG chain, recombinant decorin is inhomogeneous in size and hence does not meet the criteria for human drugs [19]. The GAG chain,

however, is dispensable for most of its tumor suppressor effects, and the majority of its interactions with growth factors or RTKs occurs via binding to the protein core. Thus, synthesizing decorin without its GAG chain may be a viable option [19], and these manufacturing issues can be simply solved by site-directed mutagenesis [19, 51].

In preparation for a future clinical development, the recent years have seen multiple efforts to improve the biological activity of decorin and thus make it more attractive as a therapeutic drug. A systemically administered, targeted version of decorin core protein was developed [19, 80, 81, 181], where the enhanced core protein is obtained by fusion to a small peptide acting as an address tag that delivers decorin to inflammatory and angiogenic vasculature [80, 81]. The small peptide tag named "CAR" (after its sequence CARSKNKDC) selectively homes to neoangiogenic blood vessels of tumors and regenerating tissues [19, 81, 182] and can efficiently deliver therapeutic amounts of decorin. Beside its tissue specificity, the CAR-DCN fusion protein displays enhanced inhibition of TGF-β-stimulated tumor cell proliferation and spreading [81, 183]. The CAR peptide targets only the inflammatory but not the normal vasculature in lung diseases and is suitable for delivery of pharmaceutical agents in an organ-specific fashion [182, 184–188]. These improvements in delivery and activity strengthen the rationale for applying decorin in the treatment of cancer and other pathological conditions related to angiogenesis or inflammation [19].

2.7 Conclusions and Future Perspectives

The tumor microenvironment plays a determining role in cancer development by regulating multiple processes between the extracellular matrix and tumor cells. Decorin, a prototype member of the SLRP family found in a variety of tissues, has gained recognition for its essential roles in inflammation, fibrotic disorders, and cancer. Originally discovered as a collagen-bound molecule regulating fibrillogenesis, decorin has emerged as a multifunctional and multifaceted signaling molecule whose activity repertoire is far beyond being a mere structural component of the stroma. Studies on mice with ablated decorin gene revealed that the lack of decorin is permissive for tumor development. On the same note, reduced expression or abrogation of decorin was observed in several types of cancer, suggesting that decorin tends to act as a tumor suppressor in these contexts. Moreover, when applied as a therapeutic agent, decorin effectively inhibited tumor formation, progression, angiogenesis, and metastasis in a multitude of experimental models, which raised substantial interest in decorin for clinical medicine. The antitumor activity of decorin relies on targeting a wide selection of binding partners such as growth factors, cell surface receptors, and extracellular matrix components, and its combined effects amount to potent inhibition of downstream signaling pathways of cell proliferation, migration, and angiogenesis. Through a novel regulatory mechanism, decorin was also shown to induce conserved catabolic processes such as endothelial cell autophagy and tumor cell mitophagy.

In summary, while the biological action of decorin is irrefutably complex, all its interactions lean toward curbing tumor progression. The antitumor effect is achieved partly by directly modulating tumor cells and partly by influencing the surrounding tumor microenvironment that would otherwise promote malignant transformation and tumor progression. Therefore, decorin as "a guardian from the matrix" may be a valuable tool in combatting cancer, especially those cancer types that heavily depend on RTK signaling.

The future of utilizing decorin in medicine is laden with challenges. As decorin is recognized and cleaved by multiple proteases, decorin can easily be inactivated in pathological processes. Thus, strategies of delivering intact and functional decorin protein core in therapeutically relevant quantities are on quest, and enhancements such as tissue targeting or improved efficacy will be welcome. Identification and isolation of leucine-rich repeats of decorin exhibiting distinct bioactivities could be of great interest, as engi-

neering these fragments could allow specific targeting of receptors and could be developed into adjuvant peptide therapy. Further studies should also elucidate the functional interaction between decorin and existing anticancer chemotherapeutics to reveal possible limitations of its use.

For basic research, the interactome of decorin suggests many unexplored abilities and possibilities. Future investigations may identify novel binding partners and signaling pathways or reveal previously unknown connections between cellular processes. Furthermore, as decorin is a member of the SLRP family, it is conceivable that other SLRPs share interacting partners with decorin and thus create a complex dynamic signaling network. Our increasing understanding of the interactome of decorin is only one of the recently revealed mysteries of the extracellular matrix that assists in expanding our comprehension of molecular and cellular oncology.

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3 Syndecan-1 in the Tumor Microenvironment

Adriana Handra-Luca

Abstract

Syndecan-1 along with the other three syndecan proteins is present in the varied components of the tumor microenvironment: fibroblasts, inflammatory tumor immunity-associated cells, vessels, and extracellular matrix. Epithelial and non-epithelial tumors may show stromal syndecans. The main relevance of stromal syndecans as tumor biomarker resides in the relationships to tumor features such as type and differentiation as well as to prognosis.

Keywords

Syndecan-1 · Tumor · Stroma · Microenvironment · Pathology · Microscopy · Immunohistochemistry · Fibroblast · Vessels · Inflammatory cells · Extracellular matrix · Adenocarcinoma · Squamous cell carcinoma · Morphotype · Prognosis

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3.1 Introductory Section

This chapter focuses on syndecan-1 in tumor microenvironment components as detected by immunohistochemistry. Data on the other three syndecans are also presented. The relevance for tumor biology and prognosis is discussed.

3.2 Tumor Microenvironment

The tumor microenvironment is mainly represented by the tumor stroma. The components of tumor stroma are the fibroblasts and myofibroblasts, the inflammatory cells (frequently lymphocytes and plasmocytes), the blood vessels, and the extracellular matrix (tumor-associated interstitial tissue or connective tissue). The tumor microenvironment is easily detected in epithelial tumors when abundant. In mesenchymal and hematopoietic tumors, the tumor microenvironment is scant, represented mainly by reactive inflammatory cells and vessels.

The majority of studies with regard to syndecans and tumor microenvironment focus on expression of these molecules in stromal fibroblasts and extracellular matrix. A pleiotropic role for syndecan-1 is suggested as determined by the cellular origin, location, and type: for example, tumor promoter when stromal and tumor suppressor when epithelial [8].

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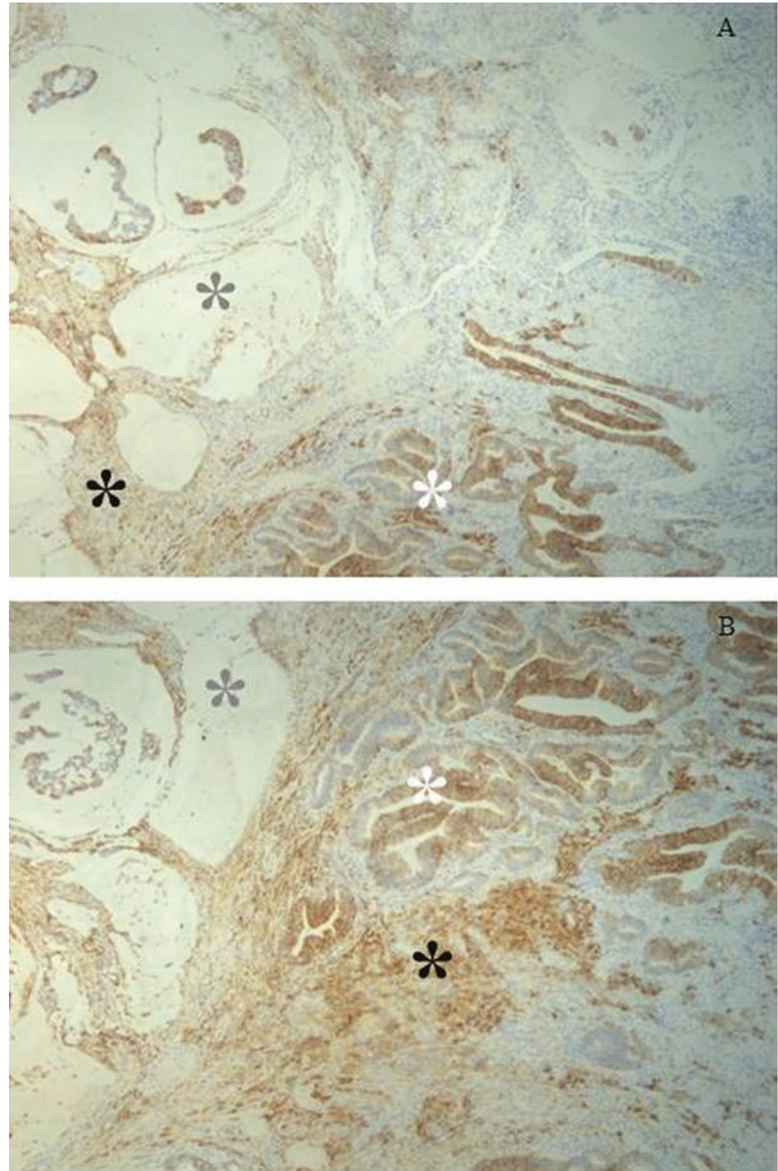

Fig. 3.1 In colon adenocarcinoma, syndecan 1 (as detected by the anti-CD138 antibody, clone MI15 Leica Biosystems) was expressed heterogeneously in the epithelial tumor component and in the stroma (asterisk white and black, respectively, gray asterisk for extracellular, stromal mucus). Immunohistochemistry: Leica system microscopy photos, original magnification ×5 (**a**, **b**)

Varied tumor types have been studied for syndecans in the tumor microenvironment, with epithelial tumors being the most frequently reported. Representative examples for syndecan-1 expression in the tumor stroma are shown in Figs. 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, and 3.9. Among epithelial tumors, glandular-type carcinomas of the gynecological and gastrointestinal systems are the most studied. Breast carcinomas have been extensively studied for stromal syndecans [4, 7, 9, 11, 12, 14, 15, 19, 20, 28]. Other studied gynecological system carcinomas are those of the endometrium [10] and of the ovary [8]. There are also reports of syndecans in gastric [32], colorectal [20], and hepatic tumor tissues [26]. Among the squamous cell carcinoma-type epithelial tumors, most reports are with regard to the tumors located in the head and neck [22] and oral regions [1] and of the skin. Lung non-small cell carcinomas [3, 20] have also been studied for syndecans as well as transitional-type epithelial tumors such as urinary bladder carcinomas [20, 31]. Less fre-

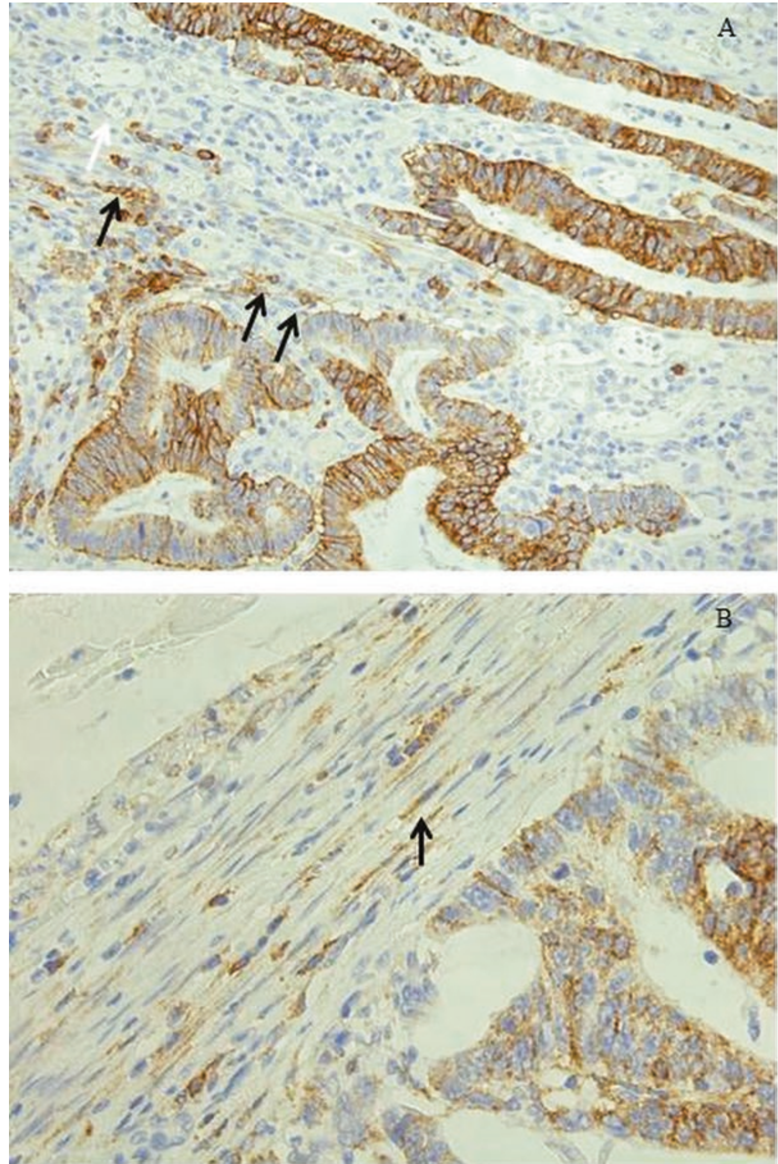

Fig. 3.2 In colon adenocarcinoma, syndecan-1 (as detected by the anti-CD13s antibody, clone MI15 Leica Biosystems) in lympho-plasmocytes at proximity to tumor glands (black arrows) as well as in stromal fibroblasts. Endothelial cells did not express CD138 (white arrow). Immunohistochemistry: Leica system microscopy photos, original magnification ×5 (**a**) and ×40 (**b**)

quent tumors such as salivary gland tumors may also express stromal syndecan-1 [2]. Syndecans are also expressed in the stroma of multiple myeloma, a non-epithelial tumor [5].

The fibroblast is considered the most important cell type in the tumor stroma. The fibroblast synthetizes, organizes, and maintains the 3D network of glycoproteins and proteoglycans known as the extracellular matrix [33]. Carcinoma-associated stromal fibroblasts are supposed to have an activated phenotype, pointed out by expression of smooth muscle markers (suggestive for contractile proteins), by an enhanced proliferative and migratory potential, by an altered gene profile, and by a contribution to an altered extracellular matrix architecture [33].

Syndecan expression in stromal components is considered the result of both tumor induction and tumor shedding/shift [19, 20, 30]. Shed syndecans is considered to retain biological activity [27]. The use of varied syndecan-1 clones for immunohistochemistry, that is, a

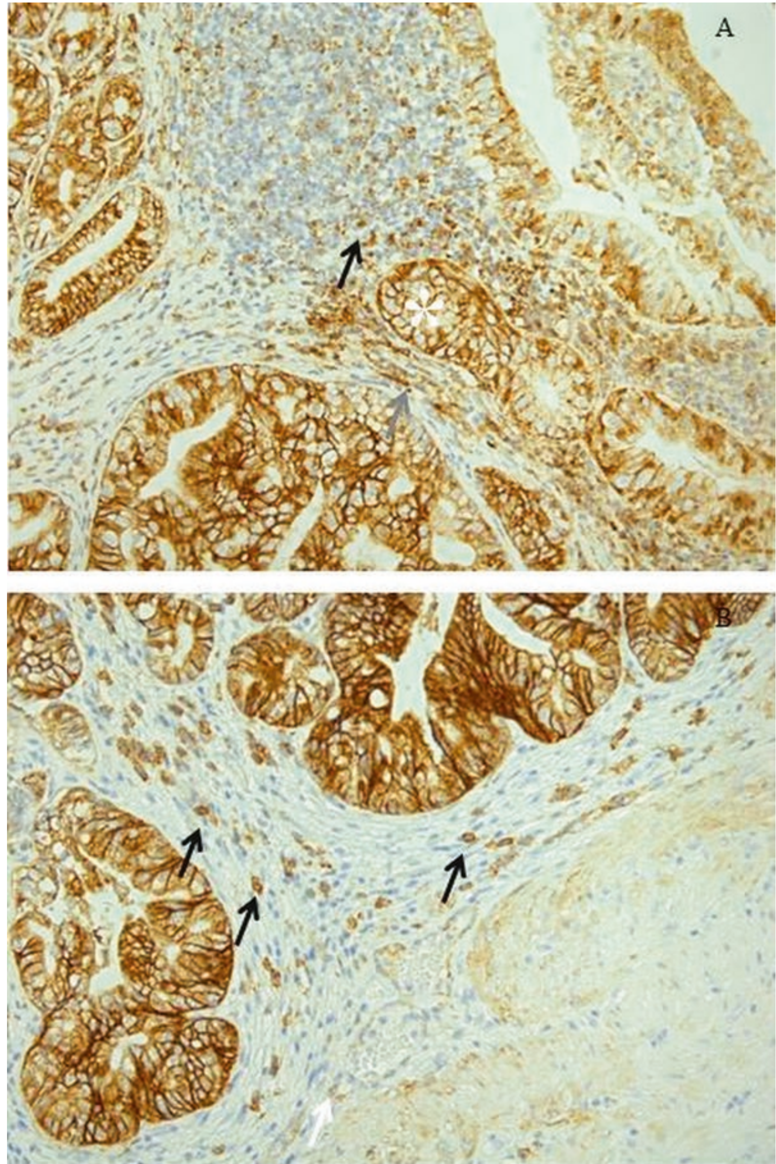

Fig. 3.3 In gallbladder adenocarcinoma, syndecan-1 (as detected the anti-CD138 antibody, clone MI15 Leica Biosystems) was expressed heterogeneously by stromal lympho-plasmocytes; black arrows). Endothelial cells also expressed CD138 (white arrow). Immunohistochemistry: Leica system microscopy photos, original magnification ×10 (**a**) and ×20 (**b**)

clone against the cytoplasmic domain (2E9 clone) and a clone against the ectodomain (IC7 clone), suggests a possible stromal synthesis of syndecan-1 as reported for human ovarian tumors [8]. Further, syndecan-1 induction in fibroblasts is required for their mitogenic effect on breast carcinoma cells [30]. Elevated syndecan-1 in mesenchyme-derived stromal/tumor-associated fibroblasts and decreased level in the epithelial cells in infiltrating carcinoma, as observed on tissue sections, resemble to what observed during embryonal morphogenesis for the condensed mesenchyme. These expression patterns in mammary carcinomas may be considered as an oncofetal reactivation of an epithelial-mesenchymal pathway in tumors [19]. Further, syndecan-1-positive fibroblasts induce carcinoma cells to form branching irregular clusters resembling infiltrating carcinoma. For the epithelial-stromal signaling, direct carcinoma cell-fibroblast contact is required. The diffusion range of released syn-

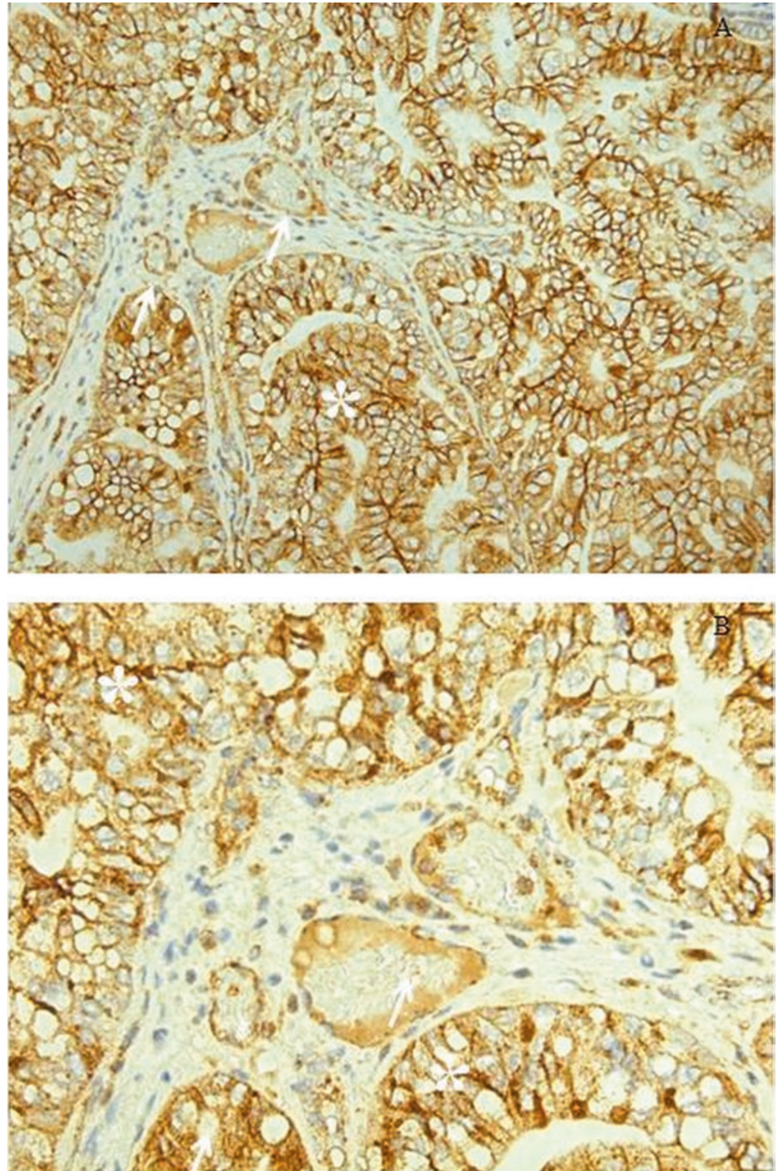

Fig. 3.4 In gallbladder adenocarcinoma, syndecan-1 (as detected by the anti-CDI38 antibody, clone MI15 Leica Biosystems) was expressed by endothelial cells. Stromal fibroblasts also expressed (heterogeneously) CD138. Tumor cells expressed strongly and diffusely CD138 (white asterisk). immunohistochemistry: Leica system microscopy photos, original magnification ×10 (**a**) and ×20 (**b**)

decan-1 ectodomain may be limited by binding to pericellular matrix components. The disruption of syndecan-1 shedding could be an opportunity for therapeutic intervention. Of interest would be that syndecan-1 in breast carcinoma stromal fibroblasts promotes the assembly of an architecturally abnormal extracellular matrix permissive for breast carcinoma directional migration and invasion [33]. As suggested by coculture experimental models, syndecan-1 in stromal fibroblasts stimulates breast carcinoma growth and angiogenesis and is considered to alter extracellular matrix composition and architecture. Altered extracellular matrix fiber architecture promotes directional migration of breast carcinoma cells [33]. Of note would be that genotoxic radiation can provoke premature senescence in breast stromal fibroblasts. An autocrine TGF-beta loop is formed leading to syndecan-1 overexpression [13].

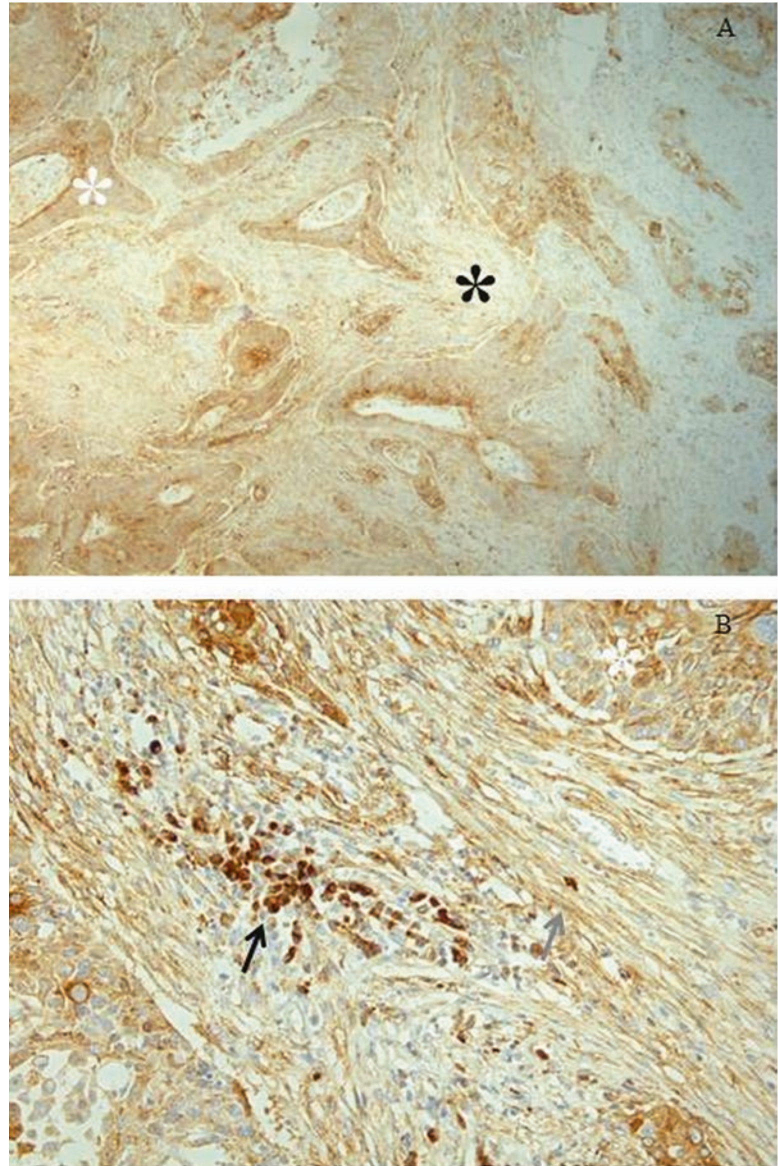

Fig. 3.5 In lung squamous cell carcinoma (non-small cell carcinoma). syndecan-1 (as detected by the anti-CDI38 antibody, clone MI15 Leica Biosystems) was heterogeneously expressed in the tumor and stromal components (white and black asterisks). CD138 was expressed strongly in lympho-plasmocytes (black arrows) while moderately in stromal fibroblasts (gray arrow). Immunohistochemistry: Leica system microscopy photos, original magnification ×5 (**a**) and ×40 (**b**)

3.3 Syndecan-1 and Tumor Microenvironment: Detection Methods

The main and most important detection method for syndecans in tumors is immunohistochemistry. This technique, performed on tissue sections, allows the detection of syndecan protein expression not only in tumor cells but also in the tumor microenvironment components: fibroblasts, endothelial cells, inflammatory cells, and vessels. Cellular syndecan protein expression location may be evaluated whether nuclear, cytoplasmic, or membrane. The type of intracellular expression can also be assessed: cytoplasmic dot-like or diffuse, membrane, continuous or discontinuous. Nucleolar staining can also be detected [26]. Most reports in the medical literature focus on tissue expression of syndecan-1. Antibody clones used for the detection of syndecan-1 are B-B4 clone (detecting the syndecan-1 ectodomain) [1, 4, 6, 8, 9, 12, 14, 20, 22, 28, 32], MII5 [17, 23, 31],

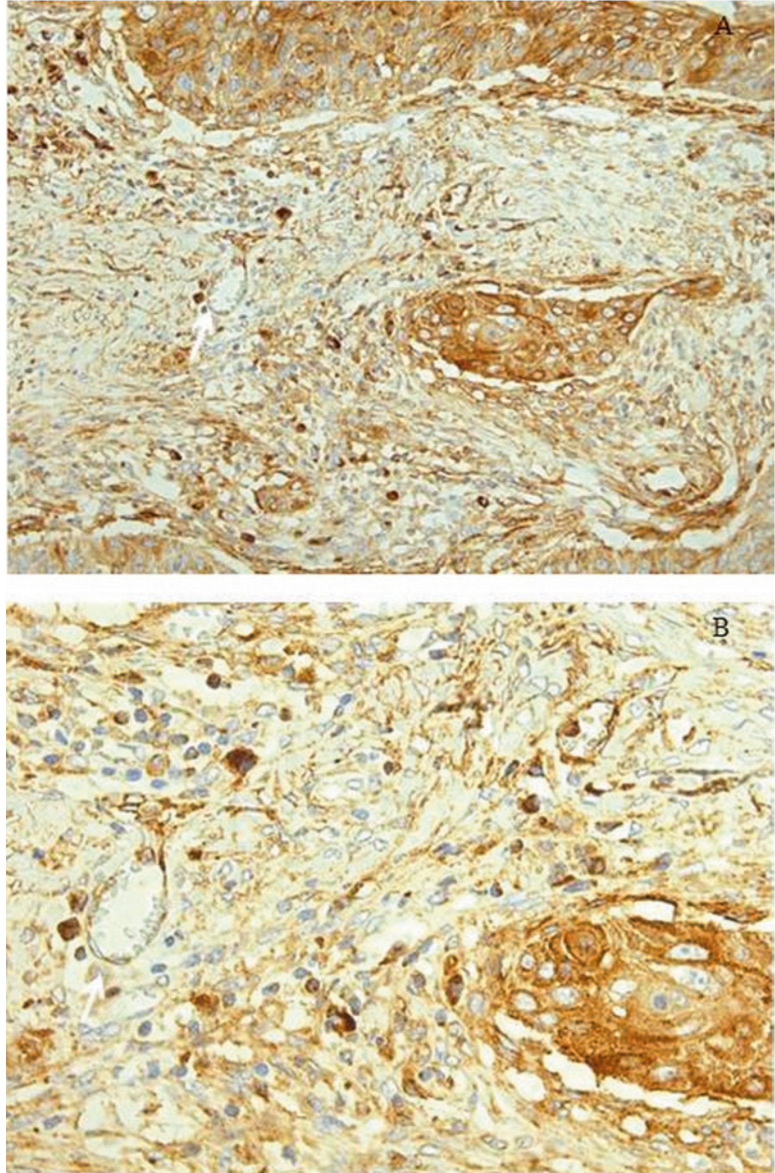

Fig. 3.6 In lung squamous cell carcinoma (non-small cell carcinoma), syndecan-1 (as detected by the anti-CD138 antibody, clone MI15 Leica Biosystems) was heterogeneously expressed in endothelial cells (white arrow). Immunohistochemistry: Leica system microscopy photos, original magnification ×5 (**a**) and ×40 (**b**)

B-A38 [3], 2E9 (detecting the epitope of the cytoplasmic domain of syndecan-1 and syndecan-3) [26], C-20 [18], 281–2 [19], M7228 [16], JASY1 [11], DL101 [21], and monoclonal IgG1 Santa Cruz [15]. The other syndecan proteins can also be detected on tumor tissue sections by immunohistochemistry. The clones used for the detection of syndecan-2 are 10H4 [26] and ZMD308 [1]. The clone used for the detection of syndecan-3 is IC7 (detecting the ectodomain), while the clones used for detection of syndecan-4 are 8G3 [8, 26] and 5G9 [12]. The fibroblast morphotype is confirmed by the presence of other proteins such as vimentin and alpha-smooth muscle actin and by the lack of cytokeratin [19, 20].

Other methods of tissue in situ detection of syndecan molecules in human tumors are immunofluorescence staining [12, 16, 18, 29], in situ hybridization [20], and immunoelectron microscopy [26]. Flow cytometry can also be used [5]; however, this method does not allow an intratissue localization of syndecan molecules.

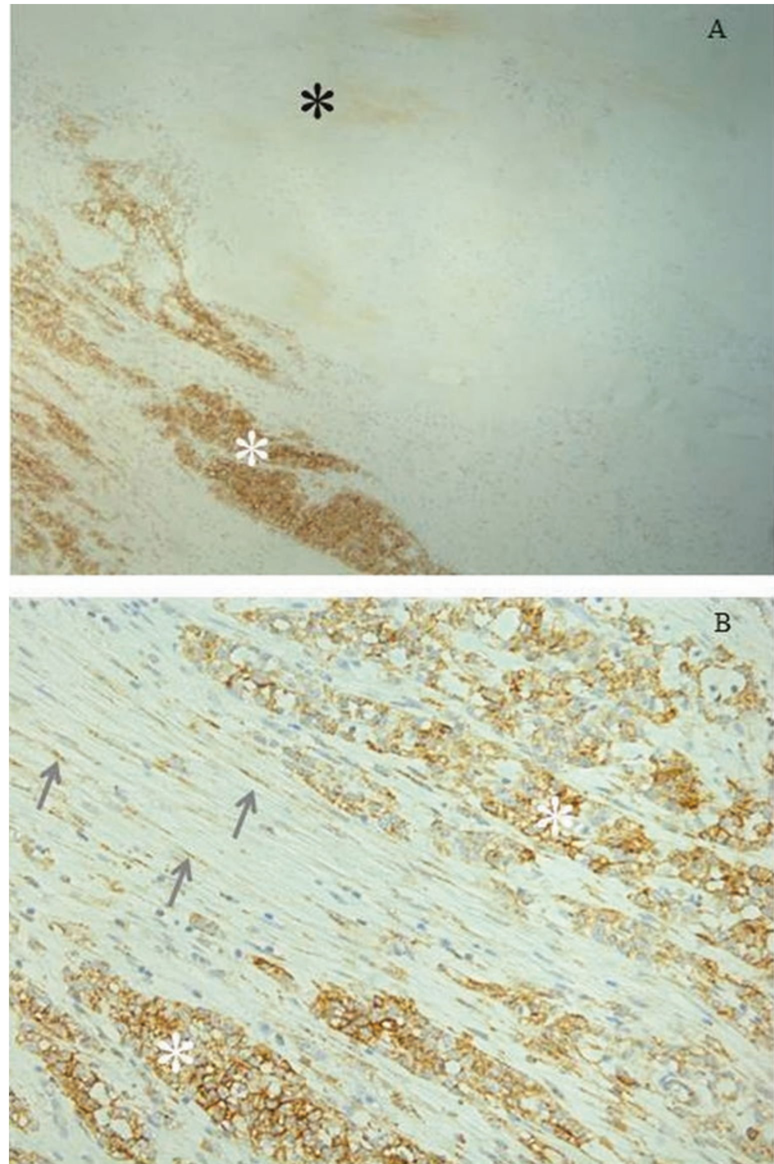

Fig. 3.7 In pancreatic solid and pseudo papillary neoplasia, syndecan-1 (as detected by the anti-CD138 antibody, clone MI15 Leica Biosystems) was expressed heterogeneously in the extracellular matrix (black asterisk). The tumor cells expressed strongly syndecan-1 (white asterisk) as well as stromal fibroblasts (gray arrow). Immunohistochemistry: Leica system microscopy photos, original magnification ×2.5 (**a**) and ×20 (**b**)

3.4 Syndecan-1 and Tumor Microenvironment: Fibroblasts

Syndecan-1 is expressed by stromal fibroblasts in 9.3–83% of human breast carcinomas [4, 7, 9, 11, 12, 14, 15, 19, 20, 28]. Tumoral stromal syndecan-1 is significantly increased as compared to normal breast tissue [15]. Benign tumors such as fibroadenomas and cystadenoma phyllodes do not show stromal fibroblast syndecan-1 [28]. In the series reported by Barbareschi et al. [4], stromal expression of syndecan-1 (fibroblast and stroma syndecan-1) was heterogeneous. In 129 of the studied tumors, more than 10% of the stroma was stained. A complete lack of syndecan-1 expression was detected in 37% of the breast carcinomas, while a strong intensity staining was detected in 32%. The most intense staining was in those tumors with dense desmoplastic stroma. In the series reported by [20], stromal syndecan-1 was of increased intensity as compared to

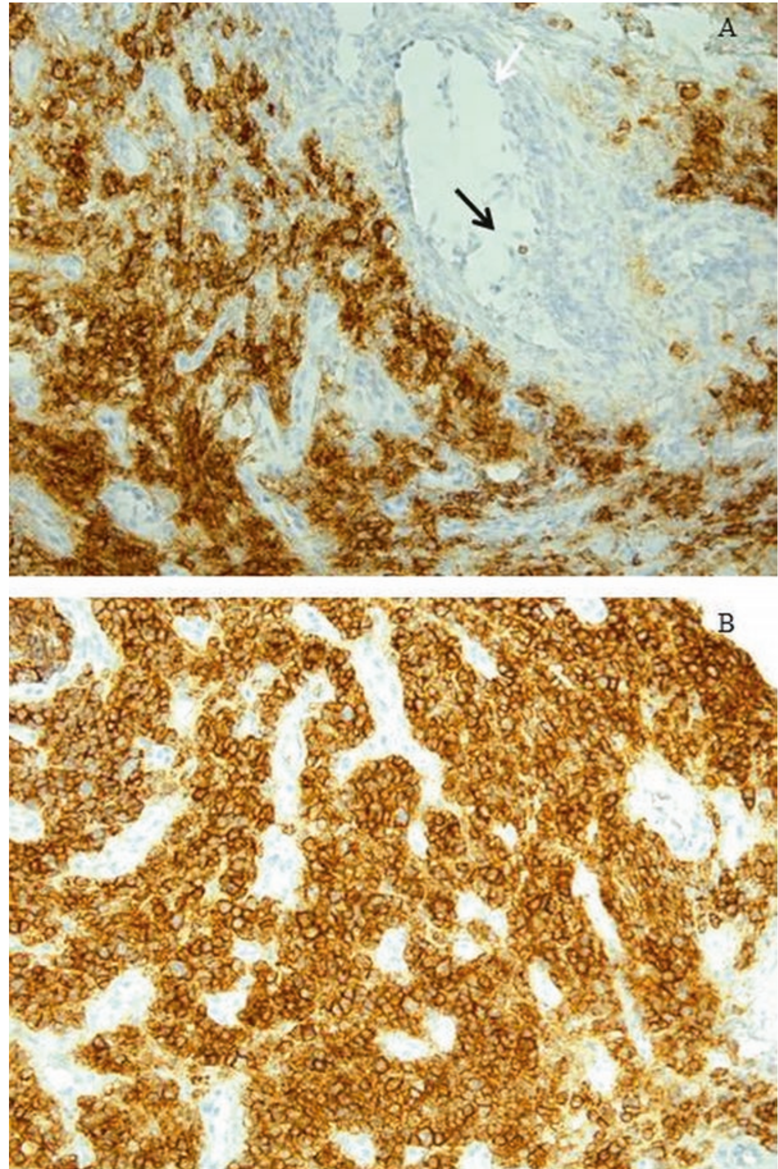

Fig. 3.8 In multiple myeloma, syndecan-1 (as detected by the anti-CD138 antibody, clone MI15 Leica Biosystems) was not expressed in the stromal vessels (white arrows). One CD138-positive plasmocyte was observedd in a vessel lumina. Tumor cells expressed diffusely and strongly CD138. Immunohistochemistry: Leica system microscopy photos, original magnification ×20 (**a**, **b**)

tumoral, epithelial syndecan-1. More recently, Kind et al. [11] detected, in the 58.1% of tumors positive for stromal syndecan-1, two "stromal syndecan-1" patterns: peritumoral and diffuse. In the series reported by Lendorf et al. [12], the highest stromal cell syndecan-1 expression was in invasive carcinomas, ductal and lobular. In the latter series, stromal syndecan-1 was confined to fibroblasts adjacent to tumor cells [12]. Of note would be that in breast carcinomas, increased stromal syndecan-1 is also detected after ionizing radiation. Increased syndecan-1 appears also at periphery of senescent Sudan Black-positive (senescent) cells [13].

In human endometrial cancer [10], another type of glandular-type carcinoma, stromal syndecan-1 expression is of heterogeneous intensity. More than half of endometrial cancers show strong or moderate expression. In human ovarian tumors [8], stromal syndecan-1 is detected not only in benign tumors but also in borderline and malignant tumors. However, the highest stromal

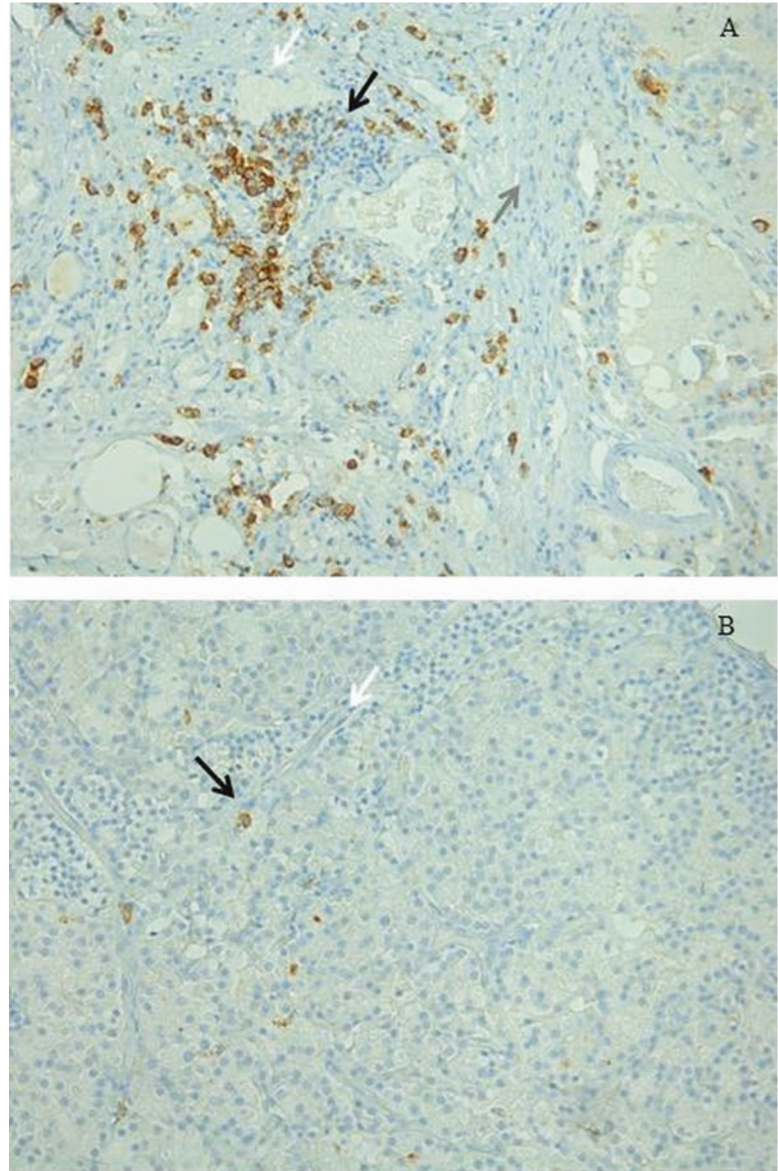

Fig. 3.9 In thyroid and parathroid (A and B, respectively) adenomas, syndecan-1 (as detected by the anti-CD138 antibody, clone MI15 Leica Biosystems) was expressed heterogeneously by stromal lympho-plasmocyte (black arrows). Endothelial cells and fibroblasts did not express syndecan-1 (gray and white arrows, respectively). Immunohistochemistry: Leica system microscopy photos, original magnification ×20 (**a**) and ×10 (**b**)

syndecan-1 expression intensity is seen in invasive adenocarcinomas, confined to cells adjacent to invasive carcinoma.

In human gastric cancer [32], stromal syndecan-1 is relatively rare (9%). Stromal expression of syndecan-1 correlates with decreased epithelial syndecan-1. A more recent report by Charchanti et al. [6] indicates that stromal moderate and high syndecan-1 is detected in most gastric tumors, 99 of the 104 tested tumors. The different results between the two studies are possibly related to different evaluation methods. In human colorectal carcinomas [20], another example of digestive system tumors, diffuse stromal syndecan-1 expression can be observed. However, in the series reported by Mitselou et al., stromal syndecan-1 was detected in 56% of the tumors [21]. Syndecan-1 is not detected in stromal fibroblasts of pancreas carcinomas [20].

In human lung non-small cell carcinomas [3], comprising a mixture of histological types including adenocarcinomas and squamous cell

carcinomas, CD138-positive cells are more abundant in the stroma than in the tumoral epithelium. A cutoff of >25% positivity in the stroma is considered as significant. To mention would be that stromal CD138 positivity correlates to intraepithelial CD138 positivity.

In human oral carcinomas [18], stromal syndecan-1 is observed in approximately one-third of the tumors. Syndecan-1 may be detected in various stromal areas, some adjacent to the tumor cells, while others at distance. In the series reported by Mukunyadzi et al. [22], more than two-thirds (74%) of the tumors show stromal fibroblast expression. The intensity of stromal fibroblast syndecan-1 is increased for tumors with a “less cohesive” invasion pattern type (invasion as single or small groups of cells) as compared to those with a “broad cohesive” invasion pattern type. Syndecan-1 is also expressed in the stroma of rare head and neck tumors, in both benign and malignant salivary gland tumors [2]. Thirty-three percent of pleomorphic adenomas and 26% of Warthin tumors (both studied cases of basal cell adenoma and myoepithelioma) as well as 54% of adenoid cystic carcinomas and all cases of acinic cell carcinomas expressed stromal syndecan-1. The expression intensity was low in pleomorphic adenomas, basal cell adenomas, and myoepitheliomas.

In human urinary bladder carcinomas [31], stromal syndecan-1 may be detected in less than half of the tumors. Mennerich et al. [20] reported stromal syndecan-1 expression decreasing in intensity with the increase of the distance from the tumor cells. Prostate carcinoma may also show syndecan-1-positive cells in the stroma, in the PCSP (prostate cancer syndecan-1-positive) cells (29).

In myeloma, a non-epithelial tumor type, syndecan-1 is not related to fibroblasts [5].

The second type of syndecan molecules, syndecan-2, is confined to stromal cells in human benign, borderline, and malignant tumors [8]. Senescent fibroblasts in human breast carcinomas after ionizing radiation show unaltered syndecan-2 [13]. In human liver carcinomas, syndecan-2 is expressed in mesenchymal stromal cells [26]. In human oral and cutaneous squamous cell carcinomas [1], syndecan-2 is expressed in the cytoplasm of stromal cells/fibroblasts in approximately half of both tumor types (50% and 56% of oral and cutaneous squamous cell carcinomas, respectively).

With regard to syndecan-3, there are few data reported for human tumors. In liver carcinomas [26], syndecan-3 is expressed in tumor mesenchymal stromal cells of hepatocellular carcinoma and cholangiocarcinomas.

Syndecan-4 is not expressed in breast carcinoma stromal fibroblasts [19] nor in ovarian epithelial tumors [8]. However, senescent fibroblasts after ionizing radiation in human breast carcinoma show syndecan-4 overexpression [13]. Moreover, 26% of the breast carcinomas showed stromal syndecan-4 [12]. The differences in the number of studied tumor may be one explanation for the different results.

3.5 Syndecan-1 and Tumor Microenvironment: Inflammatory Cells

Syndecan-1 is expressed by tumor immunity-associated lymphoplasmocytes in human lung non-small cell carcinomas [16] as well as in human oral and skin squamous cell carcinomas. In the latter two tumor types, syndecan-1 is expressed in the stromal inflammatory cells in approximately half of both tumor types (52% oral and 48% cutaneous squamous cell carcinomas) [1]. The number of syndecan-1-positive inflammatory cells is higher in oral squamous cell carcinomas than in cutaneous squamous cell carcinomas [1]. Syndecan-2 is expressed in inflammatory cells in less than 25% of both oral and cutaneous squamous cell carcinomas (14% and 22%, respectively) [1]. Syndecan-4 is not expressed in tumor stromal immune cells in the tested ovarian tumors [8].

3.6 Syndecan-1 and Tumor Microenvironment: Vessels

Syndecan-1 is reported in small blood vessels of breast tumors [12]. Syndecan-1 is also expressed in the endothelial lining of stromal vessels of gallbladder adenocarcinoma and lung squamous cell carcinoma (Figs. 3.3, 3.4, 3.6, and 3.7).

With regard to the other syndecan proteins, syndecan-2 is expressed weakly in vessels of hepatocellular carcinomas and cholangiocarcinomas. The precise location, whether endothelial or pericyte, is difficult to determine even at high-magnification optical microscopy [26]. In ovarian tissues, syndecan-3 expression in tumor stromal vessels is stronger than in normal ovarian vessels. Syndecan-3 can be detected in the vessel walls of the stroma of benign and malignant ovarian tumor [8] and in breast tumor microvessels [19].

3.7 Syndecan-1 and Tumor Microenvironment: Extracellular Matrix

To mention would be that, in several studies, fibroblast and extracellular matrix syndecan-1 are considered together as “stromal syndecan-1.” However, only extracellular matrix expression of syndecan-1 is reported in human breast carcinomas [28]. Benign tumors do not show this expression pattern.

The stromal collagen in the majority of human oral squamous cell carcinomas (91%) and in approximately half of cutaneous squamous cell carcinomas (48%) shows syndecan-1 expression [1]. The number of syndecan-1-positive collagen fibers is higher in oral than in cutaneous squamous cell carcinomas [1].

In multiple myeloma [5], a non-epithelial tumor type, the fibrotic stroma unrelated to fibroblasts, stains intensely for syndecan-1. The aspects of the syndecan-1 extracellular matrix accumulation are likely derived from shedding of the protein. In the extracellular matrix, syndecan-1 may form a reservoir of growth factors that drive reemergence of tumor after treatment [5].

3.8 Syndecan-1 and Tumor Microenvironment: Clinical Relevance

3.8.1 Tumor Features and Syndecan-1 in the Tumor Microenvironment

The relationships between stromal syndecan-1 and tumor features and outcome favor the hypothesis of a pro-oncogenic role of stromal syndecan-1.

With regard to breast cancer, stromal peritumoral and diffuse stromal CD138 relate significantly to histological tumor type, to TNM stage components, to histological grade, to estrogen and progesterone positivity, and to triple negativity [11]. In the series reported by Loussouam et al. [17], stromal syndecan-1 relates to a high Elston-Ellis grade, while in the series reported by Lendorf et al. [12], stromal syndecan-1 correlates to tumor grade and type. In human endometrial cancer, stromal syndecan-1 is associated to a high FIGO grade [10].

Stromal expression of syndecan-1 in digestive, gastric carcinomas correlates with decreased epithelial expression as well as with an epithelial intestinal tumor histotype and with Borrmann type 1 [32]. In the series of gastric tumors reported by Charchanti et al. [6], a low stromal syndecan-1 (<10%) related to the tumor histotype (intestinal versus diffuse/mixed), to histological grade, and to tumor size. In human colorectal cancer, stromal CD138 (detecting syndecan-1) (detected in 56% of the tumors) was associated to epithelial CD138 as well as to beta-catenin and CD105 microvessel density [21].

In human squamous cell carcinomas (oral and cutaneous), there is an inverse relation between syndecan-1 in collagen fibers and in tumor center [1]. In oral squamous cell carcinomas, syndecan-1 in collagen is related to the depth of the tumor (Omar 2013). In squamous cell carcinomas of the head and neck region [22], stromal fibroblast syndecan-1 expression, occurring in almost two-thirds of tumors, shows an inverse correlation to tumor cell differentiation. In less frequent tumors such as salivary gland mucoepidermoid carcino-

mas, the stromal percentage of syndecan-1 relates inversely to grade [2]. In a second type of malignant salivary gland tumor, salivary gland adenoid cystic carcinomas, the syndecan-1 stromal intensity-percentage related to gender [2].

In urinary bladder carcinomas, stromal syndecan-1 is increased in high-stage and high-grade urinary bladder carcinomas [31]. In these tumors, stromal syndecan-1 expression is enhanced in muscle-invasive tumors as compared to non-muscle-invasive tumors [31].

3.8.2 Outcome and Syndecans in Tumor Microenvironment

Results of the recent report of Kind et al. [11] suggest that stromal-positive and peritumoral stromal-positive tumors significantly relate to a better raw survival. Exclusive stromal syndecan-1 relates to a lower 7-year recurrence-free survival than those with exclusive epithelial syndecan-1 [17]. Most metastatic breast tumors (83% of distant metastatic tumors) may show stromal syndecan-1 in the cytoplasm of stromal fibroblasts and in the collagen [23]. A better response to chemotherapy is observed for patients with tumors showing stromal syndecan-1 [9]. In another example of gynecological system tumor, in human endometrial cancer, stromal syndecan-1 is associated to lower disease-free survival and overall survival rates [10]. In ovarian cancer, stromal syndecan-1 is related with shorter progression-free survival [8].

In digestive system tumors as gastric cancer, stromal expression of syndecan-1 relates to a worse outcome [32]. In human colorectal cancer, stromal CD138 (detected in 56% of the tumors) associates with liver metastases [21].

In human lung non-small cell carcinomas [3], a high stromal CD138 (detecting syndecan-1) is associated to a tendency for better disease-free survival. In the series of non-small squamous cell carcinomas reported by Lohr et al. [16], low CD138 relates to a shorter survival. When considering only the adenocarcinoma-type tumors, a high CD138 is associated to a longer survival.

In human oral carcinomas [18], stromal syndecan-1 (in "fibroid cells") is a significant risk factor of recurrence within 24 months postoperatively. Stromal syndecan-1-positive oral carcinomas show a 36.7-fold risk for tumor-associated death within 2 years [18]. In oral squamous cell carcinomas [1], stromal syndecan-1 in inflammatory cells is linked to poor prognosis. In head and neck squamous cell carcinomas [22], increased stromal expression and decreased expression in tumor cells possibly relate to the development of metastases.

In human urinary bladder carcinoma, stromal syndecan-1 strongly correlates with poor disease-specific survival and with metastasis-free survival [31].

Among the other syndecan proteins, stromal syndecan-4 is reported to relate to older age [12].

3.9 Future Trends and Directions

Data on syndecan-1 and tumor microenvironment are heterogeneous. Stromal syndecan-1 as evaluated by immunohistochemistry is considered related to clinical outcome for several tumor types. Moreover, heparanase inhibitors and anti-syndecan-1 monoclonal antibodies seem to be promising treatment options [24, 25]. Therefore, the study of an increased number of tumors would be an important direction for future research and progress.

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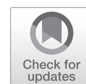

4 Versican in the Tumor Microenvironment

Athanasios Papadas and Fotis Asimakopoulos

Abstract

Versican is an extracellular matrix proteoglycan with nonredundant roles in diverse biological and cellular processes, ranging from embryonic development to adult inflammation and cancer. Versican is essential for cardiovascular morphogenesis, neural crest migration, and skeletal development during embryogenesis. In the adult, versican acts as an inflammation "amplifier" and regulator of immune cell activation and cytokine production. Increased versican expression has been observed in a wide range of malignant tumors and has been associated with poor patient outcomes. The main sources of versican production in the tumor microenvironment include accessory cells (myeloid cells and stromal components) and, in some contexts, the tumor cells themselves. Versican has been implicated in several classical hallmarks of cancer such as proliferative signaling, evasion of growth suppressor signaling, resistance to cell death, angiogenesis, and tissue invasion and metastasis. More recently, versican has been implicated in escape from tumor immune surveillance, e.g., through dendritic cell dysfunction. Versican's multiple contributions to benign and malignant biological processes are further diversified through the generation of versican-derived bioactive proteolytic fragments (matrikines), with versikine being the most studied to date. Versican and versican-derived matrikines hold promise as targets in the management of inflammatory and malignant conditions as well as in the development of novel predictive and prognostic biomarkers.

Keywords

Proteoglycans · Tumor matrix · Versican · Tumor microenvironment · Myeloid regulatory cells · Versican proteolysis · Versikine · ADAMTS · Dendritic cells · Macrophages · Biomarkers · Immunotherapy · Inflammation · Metastasis · Tumor progression

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4.1 Introduction: Structure, Isoforms, and Key Intermolecular Interactions

Versican is a chondroitin sulfate (CS) matrix proteoglycan with crucial, nonredundant roles in organ development and disease [117]. In humans, it is encoded from a single locus on chromosome 15q14.3 [50]. Its amino acid sequence is 89% identical between mouse and human [76] highlighting the highly conserved nature of this proteoglycan. The locus-encoding versican (*VCAN*, *CSPG2*) comprises 15 exons, which are arrayed over 90 kb of contiguous genomic DNA. Versican core protein consists of an N-terminal G1 domain, a C-terminal G3 domain, and CS chain-binding regions (Fig. 4.1). The G1 domain is composed of an immunoglobulin (Ig)-like module, followed by two hyaluronan (HA)-binding domains (link modules). The G3 domain of versican consists of two epidermal growth factor (EGF)-like repeats, a carbohydrate recognition (lectin-like, CRD) domain, and a complement binding protein (CBP)-like motif [124]. The expression of versican gene is regulated by a promoter that harbors a typical TATA box. Successful cloning of the gene in man, mouse, cow, and chicken has revealed the existence of at least four splice variants of versican, which differ in the size of the core protein and the number of glycosaminoglycan (GAG) chains. The central, glycosaminoglycan (GAG)-bearing domain of the versican core protein is coded by two large exons, GAG-α and GAG-β, which can be alternately spliced at exon 7 (which codes for the GAG-α region) and exon 8 (which codes for the GAG-β region). When both exons 7 and 8 are present and no splicing occurs, versican V0 isoform is formed. When exon 7 is spliced out, versican V1 is generated. When exon 8 is spliced out, versican V2 is formed. When both exons 7 and 8 are spliced out, versican V3 is formed. Since V3 contains no GAG (CS) chains and is solely composed of the G1 and G3 domains, it cannot be considered a proteoglycan, but it is frequently grouped with proteoglycans and studied as such [117, 118].

Versican is a crucial partner in extracellular matrix (ECM) assembly through key protein-protein or protein-carbohydrate interactions. One of the most studied interactions is between the amino-terminal domains of versican (G1 domain) to HA, mediated through link modules [118]. Versican interacts with diverse ECM components that are important in inflammation, such as TNF-stimulated gene-6 (TSG-6), fibulins and fibrillin, inter-alpha-trypsin inhibitor (IαI), fibronectin, tenascin-R and tenascin-C. Tenascin-R binds to versican at its C-terminal lectin-like domain (CRD) through protein-protein interactions [8]. Versican binds to fibulin-2 and fibrillin-1 through its C-terminal lectin-like domain in a calcium-dependent manner [51, 80]. Fibulin also may serve as a bridge between versican and fibrillin, forming highly ordered multimolecular structures important in the assembly of elastic fibers [117]. Versican also interacts with fibronectin, as well as collagen type I [109, 126]. Moreover, versican G3 domain can form complexes with fibronectin and vascular endothelial growth factor (VEGF). This complex was found to stimulate endothelial cell adhesion, proliferation, and migration. Disrupting the complex through anti-fibronectin antibody reversed G3's enhancing effects on endothelial cell activities [121]. Finally, versican binds to adhesion molecules on the surface of inflammatory leukocytes such as L- and P-selectins through oversulfated sequences [52, 53].

4.2 Versican and Versican Proteolysis in Embryonic Development

Versican has been implicated in cardiovascular morphogenesis, neural crest cell migration, and skeletal development. The ADAMTS protease family includes several versican-degrading members (versicanases) that are active during remodeling of the embryonic provisional matrix, especially during sculpting of versican-rich tissues [75]. Versican is cleaved at specific peptide bonds by ADAMTS proteases, and the proteo-

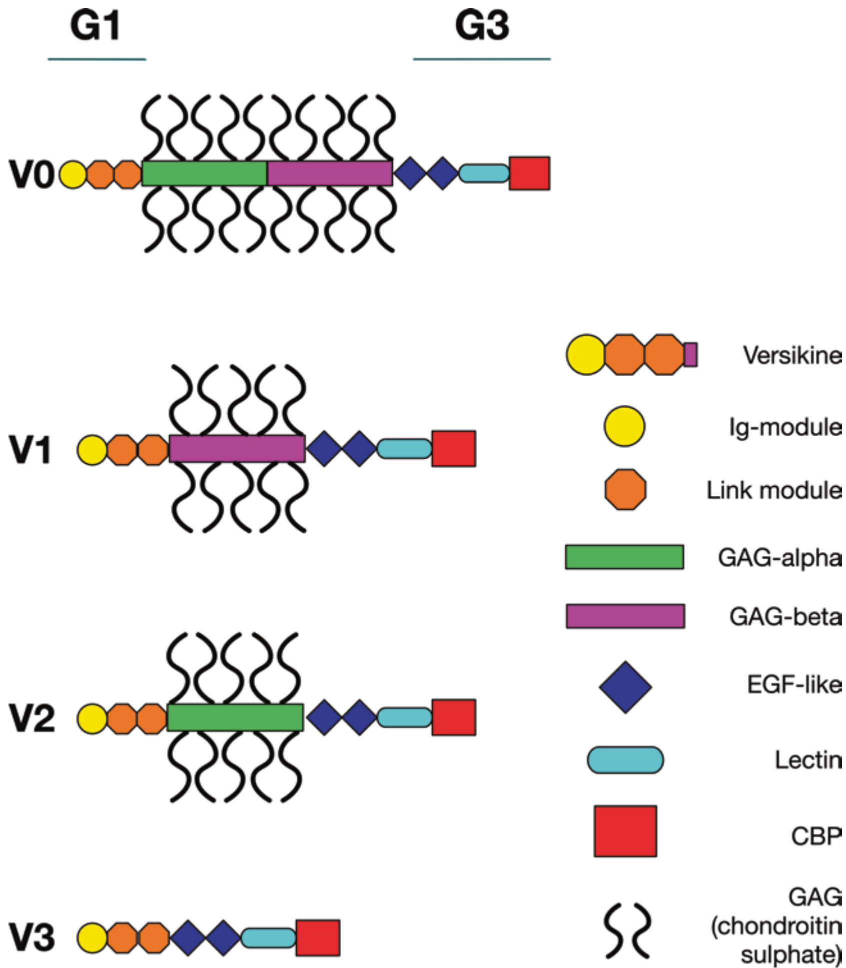

Fig. 4.1 Structure of versican, its isoforms, and its proteolytic product, versikine. Ig immunoglobulin, GAG glycosaminoglycan, EGF epidermal growth factor, CBP complement-binding protein

lytic products are detectable by neo-epitope antibodies. The developmental significance of versican's proteolytic processing has been elucidated at the sites of the most dramatic shaping of the provisional matrix such as interdigital webs, sculpting, redirection and migration of the secondary palate shelves prior to their midline fusion, resorption of cardiac jelly during myocardial compaction, and remodeling of endocardial cushions to form mature heart valve leaflets. Collectively, several studies have illustrated how proteolysis of versican deposited early in the embryo could be a regulator of morphogenetic processes during subsequent development [23, 55, 67, 88].

In cardiac development, versican is essential to the formation of endocardial cushion mesenchyme by epithelial-mesenchymal transformation (EMT). Versican proteolytic fragments generated through the actions of ADAMTS proteases can be detected in the cardiac cushions [56]. Later in development, endocardial cushions are rapidly remodeled to achieve their mature structure, and cleaved versican is broadly distributed around cushion mesenchyme cells. Congenital valve anomalies associated with accumulation of versican were seen in both *Adamts9*$^{+/-}$ mice and *Adamts5*$^{-/-}$ mice and were attributed mostly to subtle developmental alterations in extracellular matrix remodeling or defects in adult homeostasis [55, 57].

Versican proteolysis by ADAMTS9 in vascular endothelium and by ADAMTS20 in palate mesenchyme drives palatal shelf sculpting and extension. Cooperation of ADAMTS9 and ADAMTS20 contributes to secondary palate closure [23]. Reduced sculpting of the shelves and decreased growth were accompanied by accumulation of ECM and reduced cell density, with decreased cell proliferation in palate mesenchyme of the *Adamts9*$^{+/-}$ and *Adamts20*$^{bt/bt}$ mutant mice. Moreover, the palates of these embryos showed a clear reduction of processed versican as evident from reduced anti-DPEAAE staining (a neo-epitope generated by cleavage of V1 versican) [23]. *Vcan* haploinsufficiency in the *Adamts20*$^{bt/bt}$ background also led to cleft palate, demonstrating that versican was a necessary partner of ADAMTS proteases during palate closure, possibly by providing a bioactive fragment, versikine [23].

Versikine, a bioactive N-terminal fragment generated by V1 versican cleavage, is implicated in induction of apoptosis in the context of web regression. Specifically, when Affi-Gel beads were soaked in conditioned medium from HEK293 cells stably overexpressing versikine, they could induce apoptosis in ADAMTS-deficient interdigital tissues [67]. Thus, versican itself and its proteolytic derivative are essential for web regression.

4.3 Versican in Tissue Inflammation and Immunity

Versican is a major component of the inflammatory response cascade. Its production is highly regulated by inflammatory cytokine networks and, in turn, regulates downstream inflammatory mediators to amplify the response [132]. Upon extravasation in the subendothelium, leukocytes encounter ECM structures enriched in versican and HA that act as scaffold for leukocytes having an impact on their cell adhesion and subsequent retention and activation [119]. Versican interacts with receptors on the surface of leukocytes such as P and L selectins and then provides intrinsic signals that influence immune and inflammatory phenotypes [52, 53, 124, 133]. Once bound to the versican-containing ECM, leukocytes degrade the ECM to generate pro-inflammatory fragments, mostly derived from laminin, elastin, and IV collagen that further drive the inflammatory response by increasing monocyte-/macrophage-dependent secretion of proteases and pro-inflammatory cytokines [1, 6, 98, 111]. Versican, which binds to HA, can also bind to CD44 via chondroitin sulfate (CS) GAGs [52], suggesting that both versican and HA may strengthen CD44-dependent interactions and subsequent CD44-dependent signaling in inflammatory cells. On the other hand, versican binding to HA may interfere with the binding of HA to CD44 on immune cells, such as T lymphocytes [26], and attenuate the immune response. Versican proteolysis can also drive new blood vessel formation as part of inflammatory events associated with tissue repair. For instance, injection of an adenoviral vector expressing $VEGF_{164}$ into the skin induces a robust angiogenic response by increasing ADAMTS-1 and versican's proteolytic fragment, versikine [28].

Versican appears to have a role in monocyte adhesion. ECMs that did not support monocyte adhesion were deficient in versican but enriched in HA. In support of this notion, treating a monocyte-attractant ECM with an antibody against the N-terminal region of versican before adding monocytes blocked monocyte adhesion to that ECM [85]. Versican also controls inflammatory cytokine release by myeloid cells. Versican acts as a danger-associated molecular pattern (DAMP) molecule that interacts with Toll-like receptors (TLRs), such as TLR2 on alveolar macrophages, to promote production of inflammatory cytokines, including tumor necrosis factor-α (TNFα), IL-6, and other pro-inflammatory cytokines [31, 38, 114, 120].

A major source of versican production in the inflammatory milieu is macrophages. Versican gene is differentially expressed in M1 macrophages, as opposed to M2 macrophages. Matrix metalloproteinases (MMP) degrade ECM proteins [39, 43, 44]; however, ECM degradation is neither the sole nor predominant function of these enzymes. Versican produced by macro-

phages can form complexes with MMPs [65], such as MMP-9, implying possible roles for versican in controlling the activity of matrix-degrading enzymes. Such activity suggests that versican could assist myeloid cells in shaping their own microenvironment [119]. Versican can also alter the inflammatory milieu through chemokine regulation. Versican expression is elevated in CD14+ monocytes isolated from patients with systemic sclerosis, and this elevated expression is accompanied by increased expression of CCL2 [66]. Earlier studies had also shown that CCL2 binds to versican and impacts inflammation in a model of neuronal inflammation hyperalgesia [12]. In the setting of lung infection, versican and HA are increased in the lung during acute inflammation associated with *E. coli* pneumonia. Bacterial activation of TLR4 led to synthesis of versican which can itself interact with TLR4 to further modulate the inflammatory response [16].

Versican is also a crucial mediator of chronic inflammation. Versican accumulates in chronic lung diseases that involve persistent inflammation such as pulmonary fibrosis, acute respiratory distress syndrome, asthma, and chronic obstructive pulmonary disease [5, 10, 46, 72]. Versican, which is mainly secreted by fibroblasts throughout the airway tree, contributes to airway remodeling in asthma, leading to persistent airway obstruction and subsequent decline in lung function [2]. Altered deposition of proteoglycan in the asthmatic lung seems to vary between asthma phenotypes and severities [77, 84]. Interestingly, fibroblasts isolated from bronchial biopsies from asthmatic patients with the greatest degree of hyperresponsiveness produced larger amounts of versican [116]. Patients with fatal asthma had increased versican content in the internal area of large and small airways compared with controls [18]. Versican is also implicated in chronic obstructive pulmonary disease (COPD), a chronic lung condition characterized by loss of elastic fibers from small airways and alveolar walls. Fibroblasts in distal airways from COPD patients bear modifications in proteoglycan production that may contribute to disease development: there is a higher rate of versican production/accumulation compared to degradation [34]. Versican in the alveolar wall is also negatively correlated to elastin and elastin-binding protein (EBP), a molecular chaperone important in processing of elastin [69]. In versican-rich microenvironment, new formation of elastic fibers is hampered. The association between elastic fiber loss and accumulation of versican suggests that modulation of versican influences elastic fiber deposition [47, 70].

In a seminal study by the Stambas group, versican was implicated in regulation of antigen-specific, adaptive immunity. Accumulation of versican in *Adamts5*-knockout mice, which lack ADAMTS5 versicanase, causes impaired influenza virus clearance and prevents CD8+ T cell egress, leading to compromised antiviral immunity. However, when $Adamts5^{-/-}Vcan^{+/hdf}$ (versican-haploinsufficient) mice were infected with influenza virus, T cell function was restored. The authors showed that V0/V1 versican accumulation impedes migration of CD8+ T cells from draining lymph nodes to the periphery, which is critically important for the establishment of full effector function and eventual clearance of the viral pathogen [68].

4.4 Versican in Cancer

Versican is of central relevance to several hallmarks of cancer [35] and plays important roles in both malignant transformation and tumor progression (Fig. 4.2). Increased versican expression has been observed in a wide range of malignant tumors and has been associated with both cancer relapse and poor patient outcomes.

4.4.1 Source of Versican Production in the Tumor Bed

There are at least four major sources of versican production in the tumor bed: the tumor cells, the stromal cells, the tumor-associated myeloid cells, and the tumor-infiltrating lymphoid cells. Versican sources are often context-specific and not necessarily mutually exclusive.

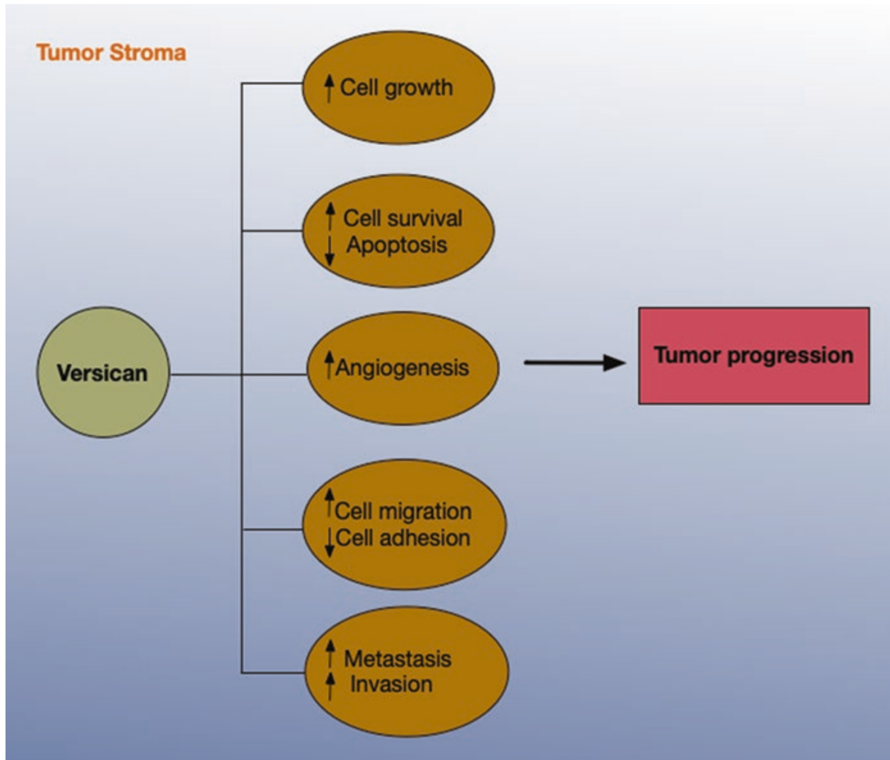

Fig. 4.2 Synopsis of the actions of versican on tumor progression

In lung cancer, versican's main source of secretion is the tumor cell. Versican secretion by the experimental lung cancer model Lewis Lung carcinoma (LLC) is necessary for metastatic spread to the lung, liver, and adrenal gland, a process that depends on TLR2-mediated myeloid cell activation and TNF-α production [58]. Tumor cells show also an elevated expression of versican in ovarian cancer [63], leiomyosarcoma [54], hepatocellular carcinoma [125], colon carcinoma [14], glioma [45], and bladder cancer [95]. Several of these studies find a direct correlation between tumor versican expression and tumor grade.

In other contexts, stromal cells constitute the main source of versican production, such as in breast cancer [17, 59, 89, 105], colon cancer [49], pharyngeal cancer [87], ovarian cancer [129], and prostate cancer [90, 96, 97]. Stromal versican is often accompanied by increased HA in the tumor bed. The increased amounts of versican and associated polysaccharide (HA) expand pericellular matrix volume and as a result distend the ECM [117]. Peritumoral versican expression is induced in stromal cells by factors secreted by carcinoma cells [13, 89, 97]. Versican, which is not expressed in normal breast tissue, gets upregulated with progressive premalignancy and frank malignancy [17]. Strong versican expression was also observed in primary pharyngeal tumors, whereas in metastatic tumors, stromal versican staining in the metastatic site was found to be significantly more intense compared to the primary tumor [87]. TGF-β has been found to induce strong stromal versican expression in breast cancer [112] as well as other types of cancer [81]. Intriguingly, TGF-β can also induce the production of versican by the tumor cells themselves, e.g., in prostate cancer [79]. In some cancers, such as endometrial and cervical cancers, tumor and stromal cells can both be the source of versican production. The combination of tumor and stromal expression of versican correlates with

shortened disease-free survival and overall survival [60].

Myeloid cells are a major source of versican production in the tumor microenvironment in certain cancer types. Studying spontaneous breast cancer murine models, Gao and colleagues showed that CD11b^{+}Ly6C^{high} monocytic cells (but not the tumor cells or other stromal cells) produces versican that subsequently promotes mesenchymal to epithelial transition and metastasis [29]. Likewise, in breast cancer, versican derived from myeloid cells is crucial for tumor metastatic potential [30]. Interestingly, co-culture of myeloid cells with bladder carcinoma cells in vitro results in upregulation of versican in the myeloid cells, suggesting that the source of versican in bladder tumors includes myeloid cells [95]. Finally, in patients with acute myeloid leukemia (AML) post-cord blood stem transplantation, macrophages were the major versican-producing cells in the bone marrow (BM) [100]. Consistent with the latter observation in the hematopoietic context, our group has demonstrated that macrophages are the major source of versican in the bone marrow of patients with multiple myeloma [42].

4.4.2 Role of Versican in Cancer

4.4.2.1 Tumor Cell Proliferation and Self-Renewal

Versican is a crucial mediator of tumor cell proliferation and, in some cases, proliferation of essential tumor-accessory components. Versican enhanced proliferation rate of melanoma cells [109]. The G1 domain of versican is thought to stimulate proliferation by destabilizing cell adhesion [127], while the G3 domain mediates proliferation through two EGF-like motifs, which play a role in stimulating cell growth [22, 130, 131]. The EGF motifs were also shown to mediate breast cancer cell self-renewal [21]. Overexpression of the versican G3 domain enhanced breast cancer self-renewal through EGFR/Akt/GSK-3β signaling and conferred enhanced resistance to chemotherapeutic drugs. Of interest, versican G3-overexpressing tumors not only showed high levels of 4B6, pEGFR, pAKT, and GSK-3β (S9P), all of which were related with tumor invasiveness, but also expressed high levels of tumor stem cell markers Sox2, Sca-1, and ALDH1 [21]. Finally, siRNA against versican isoform V1 decreased tumor cell proliferation in human glioma cells [81].

Versican also regulates the proliferation of crucial tumor-accessory components. For example, platelet-derived growth factor (PDGF) upregulates versican expression in arterial smooth muscle cells and promotes the expansion of the pericellular ECM, which is required for the proliferation and migration of these cells [24, 25, 99].

4.4.2.2 Tumor Cell Survival and Apoptosis

Genetic or epigenetic modifications in tumor apoptotic signaling machinery facilitate tumor cell survival [48]. V1 versican overexpression has been reported to cause either selective apoptotic resistance or selective apoptotic sensitization. This combination of selective apoptotic resistance and sensitivity is often seen in cancer cells. Intriguingly, murine NIH 3T3 fibroblasts overexpressing V1 versican (V1 cells) were shown to have concurrent high resting levels of p53, which confers apoptotic sensitivity and Mdm2, which is a crucial negative regulator of p53 [62]. Expression of the G1 and G3 domains of versican protects cells from apoptosis induced by death receptor ligands or cytotoxic drugs [15]. The G3 domain of versican interacts also with beta-1 (β1) integrin and protects glioma cells against free radical-induced apoptosis [122]. Furthermore, versican protects cells from oxidative stress-induced apoptosis through an enhancement of cell-matrix interactions and increased cell attachment and expression of beta-1 integrin and fibronectin [123]. However, versican has also been implicated in proapoptotic signaling. siRNA-mediated versican knockdown prevented G3-modulated cell apoptosis in human breast cancer cell lines. The somewhat contradictory roles of versican in modulating cancer cell survival and apoptosis underscore the complexity of

apoptosis regulation in tumor development and progression.

4.4.2.3 Tumor Angiogenesis

Angiogenesis is the creation of new blood vessels from the branching of preexisting ones. Tumor neo-angiogenesis provides nascent tumors with adequate oxygen and nutrients. A recent study illustrated the impact of stroma-derived versican in tumor growth and vascularization [7]. The investigators showed that the major source of versican production was the tumor stroma in B16F10 (melanoma) and LLC tumors and compared vasculature density of B16F10 tumors in *Vcan* $^{hdf/+}$ mice (haploinsufficient for versican) and wild-type littermates. A significant reduction of tumor volume as well as capillary formation in the *Vcan* $^{hdf/+}$ mice at 10 days and 13 days post-tumor inoculation compared to wild-type mice was observed [7]. Thus, genetically manipulated reduction of versican attenuates tumor angiogenesis by impairing vascular invasion into the tumor core, at the same time as exerting cell-autonomous growth regulatory effects on tumor cells [7].

In the context of the well-vascularized tumor glioblastoma, versican appears to exert a pro-angiogenic effect. The versican G3 domain enhanced angiogenesis both in vitro and in vivo. G3-expressing cells and tumors formed by these cells expressed very high levels of fibronectin and VEGF. Furthermore, the G3 domain directly interacted with fibronectin and formed a complex together with VEGF. This complex promoted angiogenesis-associated activities in endothelial cells, and its disruption inhibited these processes [134]. Consistent with the observation that G3 domain binds fibronectin, the V2 versican isoform promoted extensive vasculature formation by upregulating and binding to fibronectin [128]. Silencing fibronectin expression by siRNA abolished V2 versican's effect in enhancing vascular tube-like structure formation [128].

Pericytes also participate in normal and tumoral angiogenesis. Type 2 pericytes in particular have been shown to possess angiogenic potential and play an important role in stabilizing blood vessels in the microvasculature [11]. RT-PCR has demonstrated abundant versican message in cultured pericytes in vitro [20]. Thus, type 2 pericyte-derived versican might participate in new blood vessel formation during tumor angiogenesis.

4.4.2.4 Tumor Cell Motility and Local Invasion

Versican is associated with local tumor invasion [94]. Elevated levels of versican in the pericellular stroma is an indicator for disease relapse following surgery for clinically localized prostate cancer [90–92] and breast cancer [89, 104]. Versican has been shown to impede cell adhesion to ECM substratum, and this activity is attributed to the G1 domain: for example, versican enhances locomotion and reduces cell adhesion of astrocytoma cells through the binding of its G1 domain to hyaluronan and link protein [3, 127]. More recent studies have demonstrated that purified versican from cultured human prostatic fibroblasts inhibited adhesion of prostate cancer cells to a fibronectin substratum in vitro, highlighting the key anti-adhesive regulatory role of versican in prostate cancer [96]. Moreover, the formation of an HA/versican pericellular matrix promoted prostate cancer motility in Boyden chamber motility assays using fibronectin as a chemoattractant. Thus, prostate cancer cells in vitro have the ability to recruit versican produced by prostatic stromal cells to promote their motility [93]. These findings suggest that the formation of a pericellular sheath in vivo by prostate cancer cells utilizing versican laid down by prostate stromal cells may contribute to the development of locally invasive disease.

Silencing versican by a specific siRNA against isoform V1, but not V3, significantly decreased migration in human glioma cell lines and primary cultures in vitro [81]. Induction of stromal versican expression correlated with higher tumor grade and invasiveness in carcinomas and was associated with tumor progression [61, 101]. Elevated versican expression in tumor-associated stroma resulted in reduced numbers of intraepithelial CD8-positive T cells and enhanced cancer cell local invasion in cervical cancer [32],

whereas increased expression of CD44 and versican was associated with loss of expression of both progesterone receptor (PR) and E-cadherin [36]. Moreover, in vitro silencing of V0/V1 versican caused increased adhesion to type I collagen, laminin, and fibronectin. This was coupled with reduced cell migration in both wound-healing assays and transwell chamber assays [37].

Ovarian cancer cells have the ability to recruit stromal ECM components such as versican and HA to form a pericellular matrix which in turn promotes ovarian cancer cell motility and invasion. By using modified chemotaxis assays, treatment with versican-rich conditioned media in vitro promoted ovarian cancer cell motility and invasion and enhanced their migratory potential. However, HA oligomers (six to ten disaccharides) were able to significantly block formation of pericellular matrix by ovarian cells, as well as the increased motility and invasion induced by recombinant versican. Thus, HA oligomers could be a promising adjuvant treatment tool, administered intraperitoneally together with chemotherapy drugs to ovarian cancer patients following debulking surgery, to inhibit residual ovarian cancer cells from repopulating and invading peritoneal sites [115].

4.4.2.5 Tumor Systemic Metastasis

Versican accumulation has been associated with tumor metastasis to distant organs. Versican expression was upregulated in patients with clear cell renal carcinoma (ccRCC), and this upregulation was associated with poor prognosis and high rate of metastasis [71]. In a study of 84 matched sporadic ccRCC and normal renal tissues, patients with high versican expression had a significantly worse 5-year OS (overall survival) (p-value = 0.007) and a higher rate of systemic metastasis than those with low versican expression (p-value = 0.0139). Mechanistically, versican promoted ccRCC cell migration and invasion via MMP7 and CXCR4 [71]. In breast cancer, versican derived from CD11b+ Ly6C^{high} myeloid cells is critical in promoting metastasis to the lung in a TGF-β-dependent manner [29].

Karin and colleagues showed that versican binds TLR2 and its co-receptors TLR6 and CD14 on myeloid cells in a highly metastatic lung cancer model (Lewis Lung carcinoma, LLC). Upon activating TLR2-TLR6 complexes and inducing TNF-α secretion by myeloid cells, versican strongly enhanced LLC metastatic growth. TLR2 was absolutely necessary for metastatic growth, since no metastatic enhancement was seen in *Tlr2*$^{-/-}$ mice [58]. On the other hand, TNF-α is one of the major pro-metastatic factors produced by host myeloid cells. TNF-α can suppress the apoptosis of cancer cells and stimulate their proliferation through NF-κB activation [64]. In addition, by increasing vascular permeability [110], TNF-α can enhance recruitment of leukocytes as well as intravasation and extravasation of cancer cells. Since TLR2 is absolutely necessary for versican to exert its metastasis-enhancing abilities and TNF-α is a product of activated myeloid cells after interacting with versican, either or both of these targets could provide a useful point for anti-metastatic intervention.

4.4.2.6 Interplay Between Versican and Immune Cells in the Tumor Microenvironment (TME)

Dendritic cells (DCs) play a crucial role in the regulation of the balance between CD8+ T cell immunity vs. tolerance to tumor antigens. Cross-priming, a process which DCs activate CD8+ T cells by cross-presenting exogenous antigens, plays a critical role in generating antitumor CD8+ T cell immunity [102]. However, DC-mediated cross-presentation of tumor antigens in tumor-bearing hosts often induces T cell tolerance instead of immunity. There is accumulated evidence that the TME modulates tumor-infiltrating DCs and other antigen-presenting cells such as macrophages, leading to impairment of their function in initiating potent antitumor immunity and even promotion of tumor progression [27, 78].

Importantly, tumor-derived versican leads to DC dysfunction through TLR2 activation. TLR2 ligation not only stimulated secretion of autocrine IL-10 and IL-6 but also led to sustained

elevation of the cell-surface receptors for these cytokines, which decreased the threshold concentration required to activate STAT3. This amplification loop reprogrammed DCs to produce high amounts of IL-10 rather than IL-12 and IL-1β when stimulated with LPS, a classic proinflammatory stimulus. Thus, versican impeded immunogenic DC activation and conceivably downstream Th1 and cytotoxic lymphocyte (CTL) differentiation [107, 108]. In multiple myeloma, versican is abundantly expressed and processed in the bone marrow [42]. We have previously proposed a model in which versican activates myeloma-associated monocytes/macrophages through TLR2/TLR6 signaling, thus generating trophic IL-1β and IL-6 induction [42]. The significance of versican pathway for human myeloma is further underscored by two recent reports: first, the high-resolution analysis of the human immune microenvironment in MM showing that myeloid-derived versican transcription was very strongly associated with MM progression and loss of protective T cell stemlike (Tcf1+) memory in favor of dysfunctional/exhausted T effectors [9] and, second, the demonstration that immunosuppressive macrophages (expressing versican, ENTPD1, and STAB1) were associated with persistence of minimal residual disease post-autologous stem cell transplant for myeloma, thus promoting relapse [4].

In the setting of mesothelioma, tumor-derived versican promotes tumor progression by shaping a tumor-conducive inflammatory milieu, mainly by blunting macrophage antitumor activities [83]. Mice harboring versican-deficient tumors presented fewer tumor/pleural macrophages and neutrophils and fewer pleural T-regulatory cells, compared to the control animals. Moreover, macrophages co-cultured with versican-deficient mesothelioma cells were polarized toward M1 antitumor phenotype and demonstrated increased tumor cell phagocytic capacity, compared to macrophages co-cultured with control tumor cells [83]. Overall, the critical cross-talk created by versican among different types of immune cells leads to an immunosuppressive TME that promotes cancer progression and metastasis.

4.5 Versican Proteolysis and Versican-Derived Matrikines in Inflammation and Cancer

Regulated proteolysis of versican by ADAMTS proteases at the Glu^{441}-Ala^{442} bond of the V1 isoform is associated with robust CD8+ infiltration in MM BM [19, 41] as well as solid tumors [40]. This proteolytic event is predicted to release a 441-aa-long N-terminal fragment, *versikine* (Figs. 4.1 and 4.3). We previously showed that versikine induces IRF8-dependent interferon-stimulated genes [41]. Versikine promotes IRF8-dependent Batf3-DC [33, 73] generation from Flt3L-mobilized BM in vitro [40] and Batf3-DC density in vivo (our unpublished data, see next paragraph). Enhanced Batf3-DC at the tumor site could provide a conceptual link between versikine and CD8+ infiltration because Batf3-DC, in addition to their role in cross-presenting tumor antigen for priming CD8+ effectors, orchestrate chemokine networks that enhance intratumoral CD8+ infiltration [102].

In order to investigate the effects of versikine in DC intratumoral composition in vivo, we utilized a transplantable Ras-driven multiple myeloma model (VQ) as well as transplantable solid tumor models (LLC and 4T1 mammary carcinoma). Tumor cells were stably engineered to secrete versikine vs. empty vector control, and they were then implanted into syngeneic recipients. Versikine influenced the DC milieu in the tumor bed by increasing the density of intratumoral Batf3-DC and depleting the cDC2 (CD11c+ CD11b+) subset. Our findings highlight an unappreciated facet of immune regulation of the tumor microenvironment through matrix proteolytic fragments ("matrikines") (Papadas et al., unpublished data accepted for presentation at the American Society of Hematology, 2019). Interestingly intense versican proteolysis in the bone marrow of patients who underwent autologous stem cell transplantation for myeloma correlated with adverse outcomes despite robust CD8+ infiltration [19]. Versican accumulation in this context is likely to produce an intensely immunosuppressive micro-

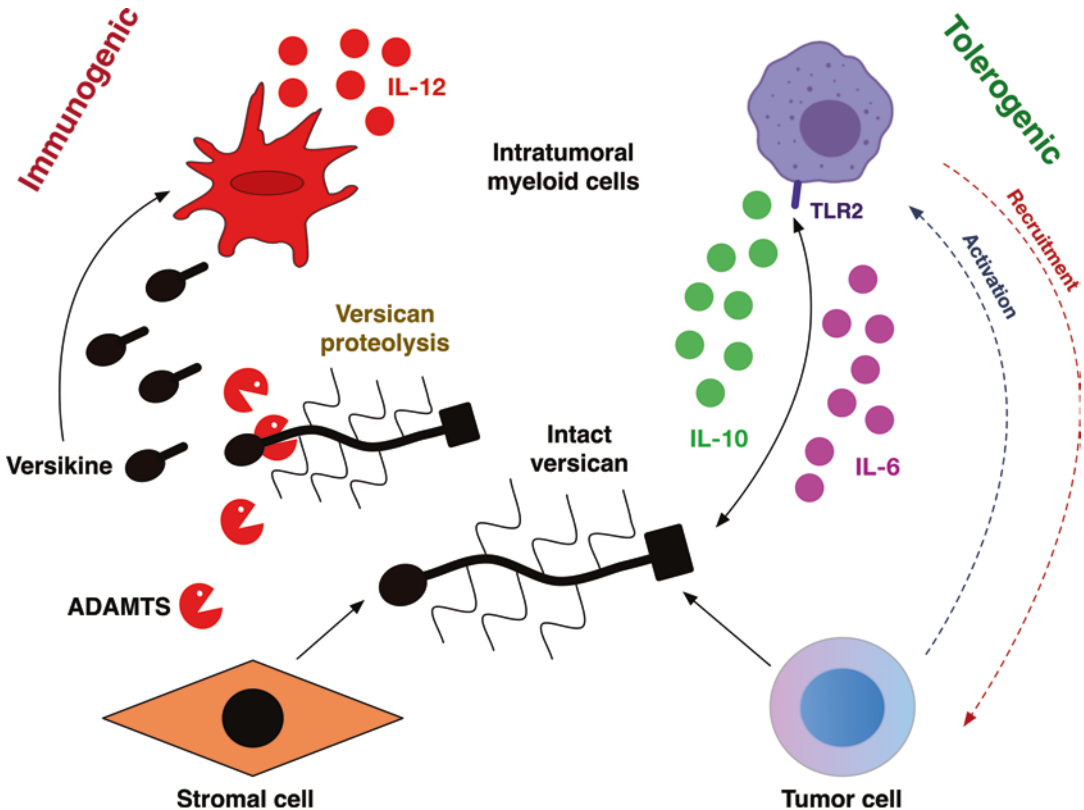

Fig. 4.3 Complex coordinated actions of versican and its proteolytic product, versikine, in the tumor microenvironment

environment that leads to effector dysfunction and impaired antitumor responses, despite the potential moderating effects of versikine signaling.

4.6 Versican: Potential for Cancer Biomarker Discovery

Versican expression correlates with poor prognosis, disease progression, metastasis, and drug resistance in cancer. The prognostic role of versican expression is tissue-specific. Versican is considered an independent and adverse prognostic marker in oral squamous cell cancer: high stromal versican expression correlates with both increased risk for disease recurrence and shortened survival for this cancer [86]. On the other hand, versican expression in the primary tumor is not an independent prognostic factor in pharyngeal squamous cell carcinoma (PSCC), although versican is more strongly expressed in the stroma of local metastases and in the earlier stages of disease in PSCC [87]. In hepatocellular carcinoma (HCC), versican expression correlates with poor prognosis, increased intratumoral macrophage infiltration, poor tumor differentiation, and a higher tumor-grade metastasis (TNM stage) [106, 125]. In colon cancer, versican expression by RT-PCR is significantly upregulated (threefold) compared to normal tissues [103]. High stromal versican expression is associated with reduced 5-year survival rates of ovarian cancer patients (44% versus 32%) [113]. Versican is upregulated in chemoresistant ovarian cancer compared to chemosensitive ovarian cancer [82]. In multiple myeloma, we recently presented the first set of data ascribing prognostic significance to the

versican proteolysis immunoregulatory pathway. We observed the somewhat paradoxical association between intense versican proteolysis and high CD8+ T cell infiltration with poor post-autologous stem cell transplant (ASCT) survival. Patients with low versican proteolysis compared to moderate/high versican proteolysis had better 2-year PFS (72% vs. 29%, $p = 0.018$) and 2-year OS (83% vs. 35%, $p = 0.006$) [19]. Thus, versican expression and/or proteolysis detection may generate powerful prognostic and in certain cases predictive (e.g., association of versican proteolysis with CD8+ T cell infiltration) cancer biomarkers [74].

4.7 Concluding Remarks and Future Directions

The versatile roles of versican in regulating cell behavior are critical in tumor development and progression. Key pathogenetic processes such as tumor proliferation, tumor cell adhesion, tumor cell survival, and apoptosis have been found to be regulated by versican. Versican supports tumor vasculature formation, tissue invasion, metastasis, and chemoresistance. Versican could act either in a cell-autonomous fashion, by having an impact on the cancer cell phenotype (proliferation, migration, and metastasis), or in non-cell-autonomous manners by influencing the tumor microenvironment, with particular bearing on tumor-associated immune cells. Versican proteolysis generates matrikines that engage in cross-talk with signaling emanating from their parent macromolecule, intact versican. Our work on multiple myeloma and relevant studies on solid tumors from other groups have provided a rationale for testing versican and versican proteolysis as potential biomarkers to predict patient outcomes. A fuller understanding of the wide array of regulatory mechanisms controlled by versican and versican-derived matrikines will strengthen the rational basis for further clinical development of tumor matrix-targeting therapies.

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5 Chondroitin Sulphate Proteoglycans in the Tumour Microenvironment

Marta Mellai, Cristina Casalone, Cristiano Corona, Paola Crociara, Alessandra Favole, Paola Cassoni, Davide Schiffer, and Renzo Boldorini

Abstract

Proteoglycans are macromolecules that are essential for the development of cells, human diseases and malignancies. In particular, chondroitin sulphate proteoglycans (CSPGs) accumulate in tumour stroma and play a key role in tumour growth and invasion by driving multiple oncogenic pathways in tumour cells and promoting crucial interactions in the tumour microenvironment (TME). These pathways involve receptor tyrosine kinase (RTK) signalling via the mitogen-activated protein kinase (MAPK) cascade and integrin signalling via the activation of focal adhesion kinase (FAK), which sustains the activation of extracellular signal-regulated kinases 1/2 (ERK1/2).

Human CSPG4 is a type I transmembrane protein that is associated with the growth and progression of human brain tumours. It regulates cell signalling and migration by interacting with components of the extracellular matrix, extracellular ligands, growth factor receptors, intracellular enzymes and structural proteins. Its overexpression by tumour cells, perivascular cells and precursor/progenitor cells in gliomas suggests that it plays a role in their origin, progression and neo-angiogenesis and its aberrant expression in tumour cells may be a promising biomarker to monitor malignant progression and patient survival.

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The aim of this chapter is to review and discuss the role of CSPG4 in the TME of human gliomas, including its potential as a druggable therapeutic target.

Keywords

Proteoglycans · Chondroitin sulphate proteoglycans · Tumour biology · Tumour microenvironment · Brain tumours · Gliomas · Glioblastoma · Stem cells · Signal transduction · Pericytes · Biomarkers · Prognosis · Therapy · Immunotherapy · CAR-T

5.1 Introduction

Proteoglycans (PGs) are located on the cell surface and in the extracellular matrix (ECM), and to a lesser extent stored in intracellular secretory granules or pericellular spaces. They are structural molecules that are necessary for correct ECM assembly, give tissues structural integrity, and regulate cell-cell and cell-ECM interactions [1]. They bind a number of soluble factors involved in cell signalling pathways (growth factors, growth factor receptors and cytokines) and regulate various cell processes (cell motility, adhesion, proliferation, differentiation and migration) [2].

PGs, including chondroitin sulphate (CSPGs) and heparin sulphate PGs (HSPGs), regulate the activity of driver signalling pathways and crucial interactions in the tumour microenvironment (TME), and their biological functions are involved in cell development, human diseases and the tumorigenesis of various malignancies [3].

In brain tumours, CSPGs are involved in multiple oncogenic pathways within the TME [3]. In particular, CSPG4 (also referred to as neuron glial antigen 2, NG2) plays a central role in tumour cell growth and motility and the survival of different tumour types [4–8]. It promotes the sustained high-level activation of key growth and survival pathways, such as the integrin-regulated focal adhesion kinase (FAK), extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) signalling pathways. The aberrant expression of CSPGs in malignant tumours (i.e. gliomas, melanomas, triple-negative breast cancer and sarcomas) may contribute to aggressive tumour growth, resistance to conventional chemotherapy in patients with glioblastoma (GB) and melanoma, and poor patient outcomes [9, 10], thus making them potential biomarkers of these tumour subsets and promising therapeutic targets [11–16].

Finally, CSPGs play a role in vascular tissue development (they are expressed by the normal or pathological pericytes associated with angiogenesis) and in the maintenance and differentiation of multipotent stem/progenitor cells [17].

5.2 Function of Chondroitin Sulphate Proteoglycans

5.2.1 Biochemical Structure

PGs are highly anionic macromolecules consisting of a core protein covalently linked to a variable number of glycosaminoglycan (GAG) chains and types. GAGs are linear polysaccharides that make up four chemically distinct subsets: chondroitin sulphate (CS) and dermatan sulphate (DS), heparin and heparan sulphate (HS), keratin sulphate (KS) and hyaluronic acid (HA). CS is a linear anionic polysaccharide that consists of alternating disaccharide units of D-glucuronic acid and D-N-acetylgalactosamine (→4GlcAβ1 → 3GalNAcβ1→) and has various sulphonation patterns [18, 19]. The sulphate groups mainly occur at C-4 and/or C-6 of N-acetylgalactosamine and/or C-2 of glucuronic acid. CS is attached to serine residues of the core protein by means of a tetrasaccharide linkage consisting of xylose, two galactose molecules and glucuronic acid [19]. On the basis of their major sulphonation patterns, CS chains are classified as CS-A or CS-C and CS-D or CS-E depending on the presence of disulphide units.

PGs are classified into three main groups on the basis of their location: on the cell surface, in intracellular space and extracellularly secreted PGs. Each group is further classified into subfamilies based on their gene homology, core protein properties, size and molecular composition.

The structural features of GAGs condition their biological functions. The PGs containing CS/DS chains are located in extracellular space (aggrecan, versican, etc.), on the cell membrane (syndecans and glypicans) or intracellularly (serglycin). The biosynthesis of CS chains on core proteins leads to the formation of CSPGs such as aggrecan (the major PG of cartilage), versican (the PG of non-cartilaginous connective tissues), decorin and biglycan. The most abundant components of the extracellular environment in the brain are CSPGs, HSPGs and HA [20].

PGs and their GAG chains undergo extensive post-translational and post-synthetic enzymatic modifications that provide the structural diversity necessary for their biological functions.

5.2.2 Function of Chondroitin Sulphate Proteoglycans in Cell Development and Human Diseases

CSPGs have a number of biological functions that act as structural components in tissue organisation and affect the important cell characteristics of proliferation, adhesion, migration and differentiation. CSPGs interact with multiple ligands (growth factors, growth factor receptors or cytokines) and play a key role in cell signalling, cell development and tissue homeostasis, but they are also involved in human diseases and malignancies [4, 5].

Given their ability to interact with different partners (including soluble factors, membrane proteins and components of the ECM), CSPGs may control processes ranging from the ligand-mediated signalling involved in cell proliferation to cell adhesion and migration [20, 21]. In addition, the intracellular domain of some transmembrane PGs can interact with the cytoplasmic domain of key proteins and contribute to regulating intracellular signalling.

The critical role of CSPGs (both the core protein and GAGs) in cell development and growth has been demonstrated by studies of animal and human diseases [3]. Alterations in CSPG core proteins, biosynthetic enzymes or extracellular regulating enzymes are associated with developmental abnormalities and, in some cases, with tumour predisposition syndromes (Simpson-Golabi-Behmel syndrome) [22, 23].

CSPGs are involved in the response to nervous system injuries and neurological diseases. In the central nervous system (CNS), they are upregulated after injury and demyelination, and this hampers axonal regeneration and remyelination [24]. In disease models, CSPG4 (also referred as neuron glial antigen 2, NG2) deficiency leads to multiple phenotypes, ranging from delays in the production of mature oligodendrocytes to deficits in brown fat function and adult-onset obesity [25, 26]. As CSPG alterations are a normal response to CNS injury, modulating this response may promote repair or improve disease status.

5.2.3 Function of Chondroitin Sulphate Proteoglycans in the Tumour Microenvironment

In line with their multiple roles in cell growth and development, CSPGs may influence a number of aspects of tumour biology, including cell proliferation, adhesion and migration, inflammation and angiogenesis [1, 2]. CSPGs regulate the bioavailability of growth factors and cytokines and the activation of their respective receptors, thus affecting phenotypic pleiotropism, gene expression and the rates of recurrences of specific tumour types.

CSPGs play a role in tumorigenesis as a result of their direct involvement in cell functions or by modulating the activity of other effector molecules such as growth factors and cytokines. During tumorigenesis, tumour cells secrete soluble growth factors that promote cell growth and activate stromal cells to secrete effectors, thus

further enhancing tumour growth. Both tumour cells and activated stromal cells are involved in reorganising the ECM in such a way as to favour tumour cell growth, migration and invasion.

In comparison with the surrounding tissue, tumour stroma and fibrotic tissue contain abnormally high levels of CSPGs. Their aberrant expression on tumour and stromal cell membranes affects tumour cell signalling, growth and survival, cell adhesion, migration and angiogenesis. Accordingly, major CSPGs (versican, decorin and CSPG4/NG2) are overexpressed in the stroma of various malignant tumours, including GBs, melanomas, osteosarcomas, and breast, pancreatic and colon carcinomas [7, 8, 27]. In particular, CSPG4/NG2 is essential for tumour growth and malignant progression as it modulates two overlapping but distinct oncogenic pathways: receptor tyrosine kinase (RTK) signalling via the mitogen-activated protein kinase (MAPK) cascade, and integrin signalling via FAK, thus sustaining ERK1/2 activation [28].

CSPGs in the TME regulate key cell processes and have different biological effects depending on the biochemical structure of their CS chains [29]. The specificity of the interactions between the CS chains and their ligands largely depends on the sulphonation pattern, which plays a critical role in tumour progression [30]. Negatively charged CS chains interact with growth factors and store them in the ECM with gradual release into the matrix by promoting cell signalling pathways. The type and fine structure of the GAG chains attached to CSPGs are markedly affected in the context of malignant transformation as a result of the altered expression of GAG-synthesising enzymes [2].

Malignant tumours have distinctive PG profiles depending on their epithelial or mesenchymal derivation. The effects of PGs largely depend on their location, expression, stage of the disease. Recent studies have attempted to define a PG "molecular signature" of tumour progression on the basis of combined comparative genomic expression profiling and the immunohistochemical staining of tissue microarrays [31].

5.2.4 Function of Chondroitin Sulphate Proteoglycans in the Brain

PGs are abundant in the brain, where they play a role in cell development and response to neurological diseases. CSPGs and HSPGs are upregulated in the neurogenic brain regions where the regulation of growth factor signalling is critical [21, 32]. CSPGs are expressed on precursor/progenitor cells, and their presence correlates with their ability to respond to fibroblast growth factor-2 (FGF-2)-supported proliferation and epidermal growth factor (EGF)-induced migration [33].

In the CNS, CSPGs are markedly upregulated in response to injury and demyelination, thus hampering axonal regeneration and remyelination [24].

CSPG2 (versican), CSPG3 (neurocan), CSPG4/NG2, CSPG5 (neuroglycan) and CSPG6 (bamacan) are the major CSPGs of the brain, with CSPG4/NG2 being an example of a pleiotropic molecule in postnatal mammalian brain.

5.3 Chondroitin Sulphate Proteoglycan 4/Neuron Glial Antigen 2 (CSPG4/NG2)

5.3.1 The CSPG4/NG2 Gene

CSPG4 is encoded by the *CSPG4/NG2* gene. The human *CSPG4/NG2* gene is located on chromosome 15q and contains 10 exons; no alternatively spliced variants have been described [8].

CSPG4/NG2 has conserved its structural and functional properties throughout its phylogenetic evolution. Its homologues in rat and mouse share over 80% amino acid sequence identity with the human sequence, and 90% with each other. Amino acid differences among the three species occur across the full-length coding sequence of each protein, suggesting that their primary structure has been conserved during evolution [5].

CSPG4/NG2 expression is regulated by a 1585 base-pair promoter region upstream of the

translation initiation site, which contains binding sites for p300 and CREB transcription factors. At post-transcriptional level, *CSPG4/NG2* mRNA is regulated by the microRNA (miR)-129-2, which binds the 3'-UTR of *CSPG4/NG2* mRNA [34].

5.3.2 CSPG4/NG2 Structural and Functional Protein Domains

CSPG4/NG2 was originally characterised as type 1 transmembrane PG in rat in 1981 [35] and was subsequently identified on the surface of human melanoma cells using a mouse monoclonal antibody (mAb) referred to as high-molecular-weight melanoma-associated antigen (HMW-MAA) or melanoma CSP (MCSP) [36, 37].

The structural features of CSPG4/NG2 are unique among the members of the PG family [38]. Human CSPG4/NG2 can be expressed as N-linked glycoprotein (full-length core protein ~250 kDa) or N-linked glycoprotein associated with a PG component (glycosylated form ~450 kDa) and contains several glycosylation sites and three putative GAG attachment sites [9, 39]. In the Golgi apparatus, CSPG4/NG2 is decorated with chondroitin-4-sulphate, which promotes its interactions with fibronectin and α3β1 integrin, and the activation of proMMP by transmembrane matrix metalloproteinases (e.g. MT3-MMP).

The CSPG4/NG2 core protein has three main structural domains: a large extracellular domain of 2225 amino acids that account for 95% of the protein; a transmembrane domain of 25 amino acids; and a short cytoplasmic tail of 76 amino acids (Fig. 5.1) [38]. It is processed into major fragments that are associated with different functions as a result of sequential cleavage by α-secretase ADAM10 and the γ-secretase complex. Proteolytic cleavage of the extracellular domain generates a soluble 290 kDa CSPG4/NG2 ectodomain, which can be released by the cell into the ECM, and a membrane-bound C-terminal fragment (CTF, 12 kDa). The latter can be further processed by the γ-secretase complex to release an intracellular domain (ICD) of 8.5 kDa containing PDZ domains [40]. The products. The producst of the proetolytic shedding are greatly enhanced in various types of lesions (spinal cord injuries, multiple sclerosis and tumours) [41].

The structure of CSPG4/NG2 indicates that it is involved in a wide range of molecular activities, including cell proliferation, migration, adhesion, and metastases and neuromodulation (Fig. 5.1). The extended extracellular domain intervenes in extracellular or pericyte-related interactions [42] and in the regulation of the neuronal network [43]. This domain binds CS chains, interacts with components of the ECM such as collagens II, V and VI [44], and forms signalling complexes with galectin-3 and α3β1 integrin possibly by acting as a co-receptor (Fig. 5.1) [45].

The intracellular domain interacts with ERK1/2 and protein kinase C-alpha (PKCα), both of which regulate proliferation, migration, invasion, cytoskeletal reorganisation, survival and chemoresistance and modulate the neuronal network [46, 47]. The mechanism underlying these functions is the CSPG4/NG2-dependent activation of α3β1 integrin regardless of whether the two molecules are expressed in the same cell or in two different cells [48]. Proliferation is enhanced by FGF/FGF receptor (FGFR) signalling via Ras and ERK, whereas survival is increased by means of PI3K/Akt signalling [48]. A transmembrane cysteine residue at C2230 is responsible for the enhanced directional motility promoted by FAK signalling [49].

The cytoplasmic domain of CSPG4/NG2 has various structural features. The four carboxyl terminal residues comprise a PDZ domain binding motif that binds the PDZ domain of scaffold proteins: i.e. syntenin-1, multiple PDZ-type adaptor proteins (MUPP1), and glutamate receptor interacting protein (GRIP). Syntenin-1 mediates interactions between CSPG4/NG2 and the actin cytoskeleton that promote tumour cell migration, whereas the presence of GRIP suggests that CSPG4/NG2 plays a role in synapse formation [50, 51]. Differential phosphorylation by PKCα (Thr^{2256}) and ERK1/2 (Thr^{2314}) respectively contributes to glioma cell motility and proliferation [46]. Finally, a proline-rich segment in the cyto-

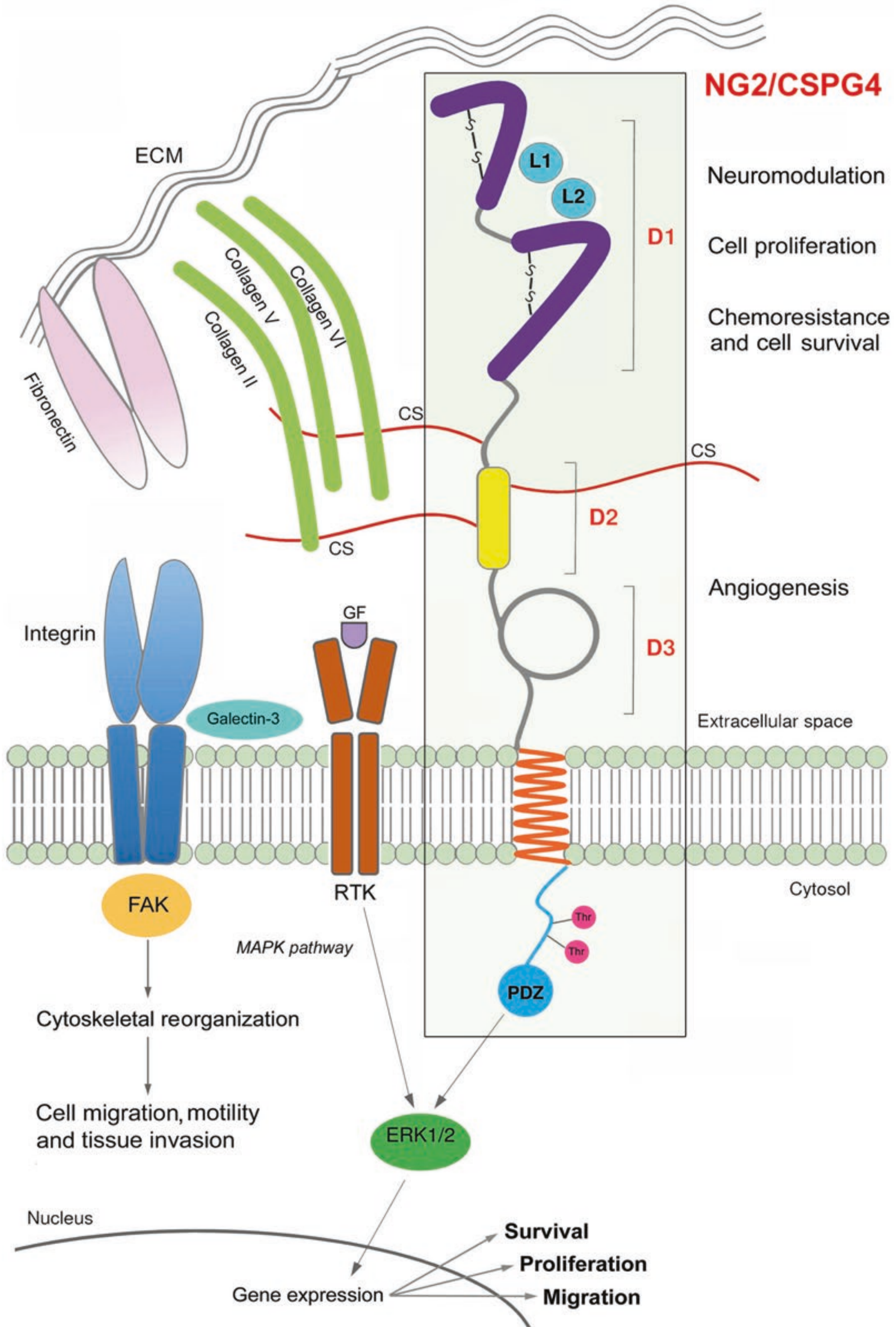

Fig. 5.1 Structure and functions of chondroitin sulphate proteoglycan 4 (CSPG4/NG2). CSPG4/NG2 is a type 1 transmembrane protein composed of an extensive N-terminal ectodomain (amino acids 1-2221), a small

plasmic domain may favour additional protein-protein interactions.

A soluble CSPG4/NG2 fragment shed by tumour cells or tumour-associated pericytes can stimulate endothelial cell (EC) migration to the TME by interacting with galectin-3 and α3β1 integrin on the endothelial surface in gliomas [45].

5.3.3 CSPG4/NG2 Expression Pattern

In terms of tissue distribution, CSPG4/NG2 is expressed in more than 10 different adult tissues (mainly the brain, the gastrointestinal tract and endocrine organs), most of which show a poor correlation between transcript and protein levels [10].

CSPG4/NG2 can be detected in >50 cell types, including chondroblasts, osteoblasts, keratinocytes, smooth muscle (SM) cells and microglia/macrophages [10, 52, 53]. Up- and down-modulation allows it to be expressed by immature progenitor cells in developmental lineages of epithelial and mesenchymal origin [10, 17, 38]. It is not expressed by multipotent stem cells, but it is upregulated in stem cells during the initial phases of commitment to a particular cell lineage. Partially committed progenitors that are still proliferative and motile and have retained a degree of developmental plasticity express high levels of CSPG4/NG2 until their terminal differentiation, when CSPG4/NG2 is turned off. The expression of CSPG4/NG2 on immature progenitor cells indicates its contribution to processes such as cell proliferation and motility that are critical for progenitor biology.

The re-expression of CSPG4/NG2 by tumour cells is associated with cell proliferation and motility and may be regarded as a marker of "activated non-quiescent" cells [7].

5.3.4 CSPG4/NG2 in CNS Biology

In the developing and adult rat and mouse CNS, CSPG4/NG2 labels oligodendrocyte-type 2 astrocyte (O2A) progenitor cells [54, 55] that become oligodendrocytes or type 2 astrocytes depending on in vitro medium conditions [56].

In adult human brain, CSGP4/NG2 is expressed on the surface of oligodendroglial precursors cells (OPCs) or polydendrocytes, the fourth largest glial cell population in the CNS [57, 58] (Fig. 5.2). OPCs are highly proliferative, migratory bipolar cells that are distinct from neurons, mature oligodendrocytes and astrocytes, or microglia. They are precursors to oligodendrocytes but may differentiate into protoplasmic astrocytes in the grey matter [59]. OPCs express platelet-derived growth factor receptor alpha (PDGFRα) [60], A2B5 [61] and 2′,3′-cyclic nucleotide phosphodiesterase (CNPase) [62].

CSPG4/NG2 promotes OPC proliferation and migration by acting as a co-receptor for the core protein-binding growth factors PDGF-AA and FGF-2 [25, 45, 63] and contributes to their polarity [64]. In particular, it regulates PDGF signalling by interacting with PDGFRα, which mediates OPC proliferation in response to its ligand PDGF [60] and EGFR signalling [65].

Fig. 5.1 (continued) transmembrane domain (amino acids 2222-2246) and a short C-terminal cytoplasmic domain (amino acids 2247-2322). The extracellular ectodomain contains three subdomains: domain 1 (D1), domain 2 (D2) and domain 3 (D3). D1 is a N-terminal globular domain (amino acids 1-640) stabilised by intramolecular disulphide bonds that contains two laminin G-type motifs (L1 and L2) and abundant disulphide bonds critical to maintain the tertiary structure. D2 is a central domain (amino acids 641-1590), containing 15 "CSPG repeat" motifs that are the attachment sites for CS chains, collagens II, V and VI. D2 interacts with integrins and ECM proteins and binds and presents growth factors to receptor tyrosine kinases. D3 is a juxtamembrane globular domain (amino acids 1591-2221) containing binding sites for galectin-3 and β1 integrins and proteolytic sites for CSPG4/NG2 cleavage. The cytoplasmic tail interacts with different proteins and functions as a phosphoacceptor site for the extracellular signal-regulated kinase 1/2 (ERK1/2). The PDZ domain is involved in protein scaffolding functions. CSPG4/NG2 activates two major cellular signalling cascades: the mitogen-activated protein kinase pathway, through the receptor tyrosine kinase-ERK1/2 axis, and the integrin/focal adhesion kinase (FAK) pathway. Through these pathways, CSPG4/NG2 ultimately promotes tumour progression across a variety of cellular functions [127]

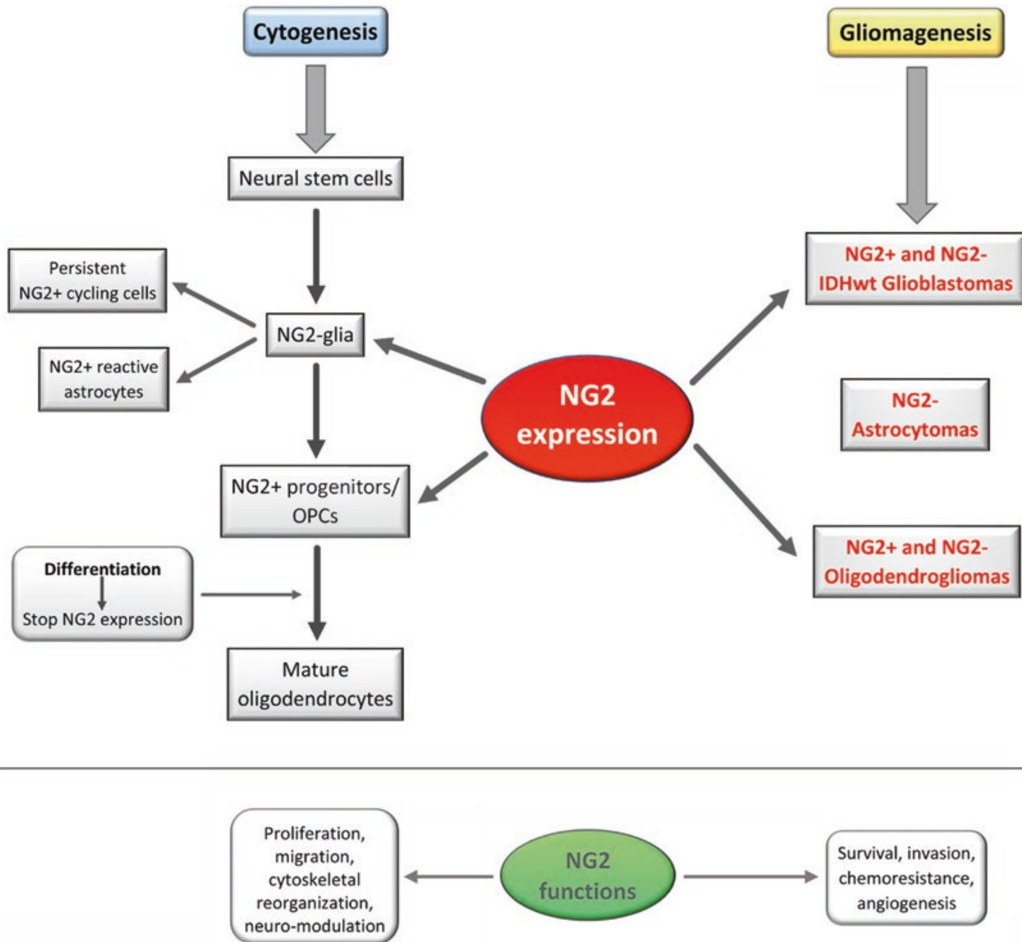

Fig. 5.2 Summary scheme of CSPG4/NG2 expression during neurogenesis and gliomagenesis and its functions in normal and pathogenetic mechanisms of the central nervous system (CNS). CSPG4/NG2 expression is detectable in subsets of normal glial cells in developing and adult CNS. It is not expressed by multipotent stem cells but is upregulated in the NG2-glia and in the partially committed oligodendrocyte precursor cells (OPCs) that are still proliferative and motile. Upon terminal differentiation of these progenitors into mature oligodendrocytes, CSPG4/NG2 is downregulated. It is once again upregulated in pathological conditions including malignant tumours. CSPG4/NG2 aberrant expression has been associated with gliomas where it affects tumour cell adhesion, migration, proliferation, chemoresistance and neo-angiogenesis [127]

CSPG4/NG2 is not expressed by multipotent neural stem cells in the primary and secondary germinal zones of the CNS, but it is upregulated in progenitors that originate in these zones and are committed to the oligodendroglial lineage [7] (Fig. 5.2). OPCs originate in the neuroepithelium of the spine and spread to populate the brain and spinal cord as a result of three waves of production and migration and may myelinate the entire CNS [66, 67]. They are uniformly distributed in the grey and white matter of the mature CNS: white matter OPCs proliferate and contribute to adult oligodendrogenesis, whereas grey matter OPCs are slowly proliferative or quiescent cells that mainly remain in a state of immaturity [68]. OPCs differentiate into less mobile, pro-oligodendrocytes that further differentiate into oligodendrocytes. The process of maturation until terminal differentiation is characterised by morphological changes and the expression of cell surface markers that are specific to the stage of differentiation. The expression of CSPG4/NG2

and PDGFRα is lost in favour of myelin basic protein (MBP), proteolipid protein (PLP) or myelin-associated glycoprotein (MAG) [69].

Adult polydendrocytes have intimate spatial relationships with synaptic structures and nodes of Ranvier, receive functional synaptic input from glutamatergic neurons [70] and act as a source of new oligodendrocytes for the remyelination of demyelinated axons [71]. Accordingly, their number increases at sites affected by injury, inflammation, demyelination or remyelination [72, 73].

CSPG4/NG2 may be a reliable marker of adult oligodendrocyte progenitors under normal, reactive or inflammatory conditions [72–74].

5.4 CSPG4/NG2 in Human Brain Tumours

5.4.1 The Role of CSPG4/NG2 in the Origin of Gliomas

The findings of basic and clinical experimental studies suggest that CSPG4/NG2-expressing OPCs may contribute to the origin and progression of human gliomas [8, 75–79].

Gliomas are the most frequent primary tumours of the CNS and are classified by the 2016 revised World Health Organisation (WHO) classification of CNS tumours into histologically and genetically identified entities and variants [80]. GB is the most frequent tumour in adults and is characterised by phenotypic and genotypic heterogeneity, resistance to therapy and recurrence. Despite major therapeutic advances, its prognosis remains poor because of the lack of local control and infiltrative growth pattern.

GB is characterised by aberrant signalling pathway activation [81–84], including abnormal RTK signalling through the MAPK cascade and abnormal integrin signalling following FAK activation. As CSPG4/NG2 participates in the extracellular availability of oncogenic factors and receptors, it may be responsible for aberrant RTK activity driven by alterations in receptor expression or altered ligand availability.

CSPG4/NG2-expressing OPCs participate in the development of adult gliomas [64, 85] (Fig. 5.2), and may give rise oligodendroglial or astrocytic tumours upon Ras activation and p53 depletion [86]. CSPG4/NG2, PDGFRα and Olig2 (all common markers of OPCs) are overexpressed in pilocytic astrocytomas and diffuse gliomas (oligodendrogliomas or astrocytomas) and heterogeneously expressed in GB [85, 87–89].

Aberrant activation of the PDGFRα signalling pathway has been described in malignant astrocytomas [82, 90]. *PDGFRα/PDGF-AA* overexpression, *EGFR* gene amplification and *isocitrate dehydrogenase 1* (*IDH1*) mutations are the main genetic alterations found in low-grade gliomas and secondary (IDH-mutant) GBs, with high *PDGFR-AA* expression levels and gene mutations being frequent in the Proneural subtype of GBs [84]. Moreover, CSPG4/NG2 is involved in the EGFR-PI3K-AKT signalling pathway, where it enhances the activation of the *EGFR* TK domain and, therefore, the proliferative capability of GB cells [91].

Despite its variable expression in human gliomas (Fig. 5.3), CSPG4/NG2 correlates with the malignancy grade [7, 16, 92, 93]. Between 50% and 67% of GBs, including GB-derived neurosphere (NS) cell lines, show predominant CSPG4/NG2 overexpression associated with stemness markers (nestin and vimentin, but not CD133) in tumour and perivascular cells [16, 92] (Fig. 5.3). GB patients with a high level of CSPG4/NG2 expression are characterised by shorter survival and increased resistance to radiotherapy [92].

NS cell lines show crosstalk between CSPG4/NG2+ and CSPG4/NG2− cells, with the former proliferating faster and being more aggressive in vivo. Lethal GBs can also arise from CSPG4/NG2– cells because a small minority may escape short hairpin (sh)RNA inhibition [91]. It is possible that CSPG4/NG2 acts as a sort of traffic light that modulates GB cell behaviour on the basis of environmental stimuli and favours programmed proliferation (CSPG4/NG2+ cells) or migration/invasion (CSPG4/NG2− cells) [91].

The expression of CSGP4/NG2 on reactive astrocytes in GBs may contribute to variable and focal positivity in the tumour parenchyma [94]

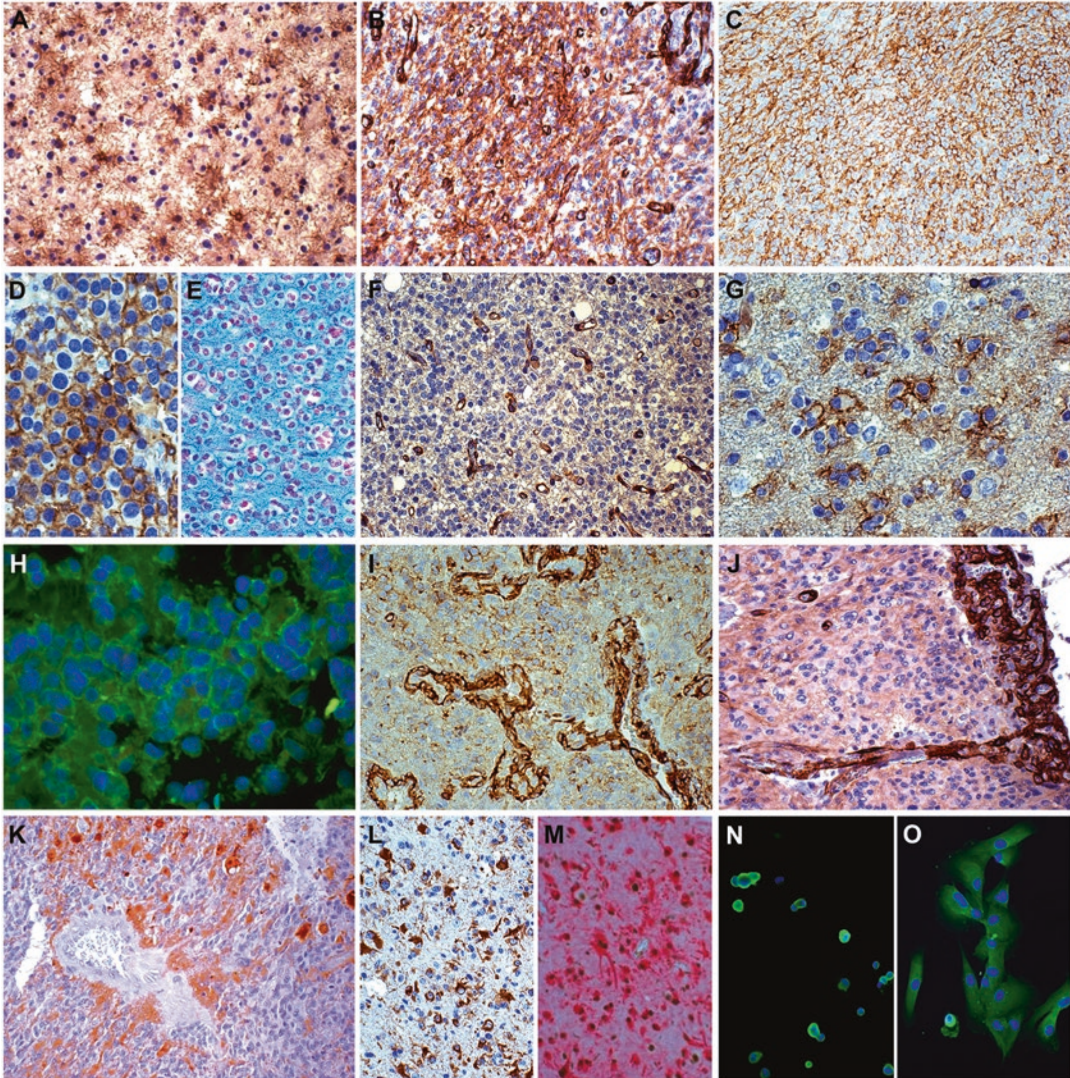

Fig. 5.3 CSPG4/NG2 immunohistochemistry. (**a**) WHO grade II astrocytoma. Negative tumour cells and CSPG4/NG2-positive reactive astrocytes; DAB, ×400. (**b**) IDH-wild type glioblastoma (GB). CSPG4/NG2-positive area; DAB, ×200. (**c**) *Id.* Diffuse CSPG4/NG2 staining on cell membranes; DAB, ×200. (**d**) WHO grade III oligodendroglioma. CSPG4/NG2-positive area with honeycomb appearance; DAB, ×400. (**e**) *Id.* Alcian Blue staining; ×200. (**f**) *Id.* CSPG4/NG2-negative tumour cells and -positive endothelial cells; DAB, ×200. (**g**) *Id.* Isolated CSPG4/NG2-positive tumour cells in infiltration area; DAB, ×400. (**h**) *Id.* CSPG4/NG2 expression in tumour cells; green immunofluorescence (IF), ×400. (**i**) IDH-wild type GB. Strong CSPG4/NG2 expression in vascular pericytes; DAB, ×200. (**j**) *Id.* CSPG4/NG2-positive vascular pericytes in glomerulus with sprouting; DAB, ×200. (**k**) *Id.* Negative tumour cells and CSPG4/NG2-positive reactive astrocytes in infiltration; DAB, ×200. (**l**) *Id.* CSPG4/NG2-positive reactive astrocytes; DAB, ×200. (**m**) *Id.* ATRX+/GFAP+ reactive astrocytes, double staining; DAB/Fast RED, respectively, ×200. (**n**) *Id.* GB-derived cell lines, neurospheres. Most cells (but not all) are variably positive for CSPG4/NG2; green IF, ×200. (**o**) GB-derived cell lines, adherent cells are weakly positive for CSPG4/NG2; green IF, ×200 [127]

(Fig. 5.3). This is in line with their origin from CSPG4/NG2+ and PDGFRα+ glial-restricted progenitors during neurogenesis and the dismal significance associated with reactive astrocytes [94]. Tumour-associated reactive astrocytes interact with tumour cells and trigger: (i) proliferation through the CXCL12 (SDF-1)/CXCR4 axis; (ii) migration and invasiveness by activating MMP-2; and (iii) survival by releasing different cytokines within the TME [95] (Fig. 5.1).

In GB, CSPG4/NG2 can also be detected on the pericytes and ECs of proliferated tumour vessels (microvascular proliferations and glomeruli), as well as on microglia/macrophages [94] (Fig. 5.3).

In addition to CSPG4/NG2, core proteins and biosynthetic enzymes are also predominantly overexpressed in human GBs [3].

CSs and GAGs were first biochemically and histochemically demonstrated in human gliomas [96–99] and rat tumours transplacentally induced using *N*-ethyl-*N*-nitrosourea (ENU) [100–102]. It was reported that CS was variably distributed on vessel walls and the cytoplasmic membranes of tumour cells mainly in regressive events and inversely correlated with dedifferentiation. Alcian blue positivity for CS is detected in isomorphic ENU oligodendrogliomas and in the peripheral part of polymorphic gliomas [101, 103].

5.4.2 Mechanisms of Signal Transduction by CSPG4/NG2 in Gliomas

CSPGs can regulate multiple steps of the tumorigenesis of human gliomas:

(i) CSPG4/NG2 interacts extracellularly through its core protein with collagen types II, V and VI, or laminin and tenascin [104], which are involved in the invasive behaviour of glial tumour cells [105].
(ii) CSPG4/NG2 acts as a co-receptor for spreading and focal contact in association with β1 integrin, a critical molecule in tumour cell adhesion and migration [106].
(iii) β1 integrin is the most abundant integrin subunit in gliomas and favours invasiveness [107].
(iv) Actin (the intracellular binding partner of CSPG4/NG2) triggers cell motility and development [50].
(v) CSPG4/NG2 enhances the activity of PDGF-AA, a well-known mitogen for in vitro glioma cell growth [108], and its α receptor signalling pathway in SM and O2A cells [60, 109].
(vi) CSPG4/NG2 promotes α3β1 integrin activation by triggering the downstream activation of FAK and PI3K/AKT signalling and increasing glioma cell proliferation, motility and survival (Fig. 5.1) [48].
(vii) CSPG4/NG2 regulates the expression of intercellular adhesion molecule 1 (ICAM-1), an essential protein for leukocyte adhesion and transmigration in pericytes and GB tumour cells [110].

5.4.3 CSPG4/NG2 in Blood Vessel Development

The progression of solid tumours is triggered by factors that are intrinsic to tumour cells and by extrinsic factors present in the TME. The effects of stromal factors allow tumour cells to spread to distant sites, whereas the interactions of tumour cells and components of the TME support tumour growth, progression and neo-angiogenesis.

CSPG4/NG2 is involved in blood vessel development under normal and pathological conditions, including tumours. It is expressed in the vasculogenic and angiogenic neo-vasculature in which crucial crosstalk between ECs and mural cells is mediated by various growth factors, including PDGFR-AA and -BB [111]. The latter intervenes at different times during vascularisation and affects ECs and mural cells in the developing neo-vasculature. A tube formation has been described in the absence of ECs but in the presence of CSPG4/NG2− and PDGFRα-expressing cells [112].

The surfaces of perivascular cells (including ECs in normal brain vessels [112, 113] and peri-

cytes of proliferated tumour vessels in malignant gliomas) express CSPG4/NG2 [114, 115] (Fig. 5.3), which is upregulated in pericytes of the neo-vasculature, downregulated in quiescent vasculature and absent or undetectable in stable healthy human adult brain vessels [116]. Mice lacking CSPG4/NG2 have defective vasculature [117].

CSPG4/NG2 and PDGFRβR, which is responsible for the ability of pericytes to respond to PDGFR-BB [118], are the most reliable markers of activated pericytes [112]. Their combined use allows their contributions to neo-vascularisation to be detected.

Vascular hyperplasia, which involves the aggressive recruitment of pericytes and ECs, is a distinctive feature of malignant gliomas, particularly GB (Fig. 5.3). Pericyte subsets show differential CSPG4/NG2 expression in GBs [119], and only one specific type of pericyte expressing both CSPG4/NG2 and nestin is thought to be recruited during tumour angiogenesis [120].

In the developing human brain and in brain tumours, NG2/CSPG4 favours the motility and angiogenesis of perivascular cells by means of galectin-3 and α3β1 integrin [45] (Fig. 5.1). Reduced pericyte interactions with ECs in PC-NG2ko mice leads to the loss of the pericytic activation of β1 integrin signalling in ECs, whereas reduced pericyte-EC interactions in Mac-NG2ko mice reduces macrophage recruitment by 90% [52].

Most of the pericytes in GB derive from GB stem cells (GSCs), and their selective deprivation disrupts the neo-vasculature and inhibits tumour growth. GSCs reside in the perivascular niche (PVN), are supported by trophic factors from the vasculature and may undergo mesenchymal differentiation giving rise to pericytes [121, 122]. They are recruited towards ECs through the CXCL12 (SDF-1)/CXCR4 axis and are induced to become pericytes by transforming growth factor β (TGF-β) [123]. GSCs may also contribute to perivascular pericytes by actively remodelling the PVN [123–126].

Figure 5.3 shows personal findings concerning CSPG4/NG2 immunoreactivity in human gliomas and GB-derived cell lines [127].

5.4.4 CSPG4/NG2 in Non-glial Tumours

CSPG4/NG2 plays a role in the progression of multiple tumour types other than gliomas. CSPG4/NG2 overexpression in radial growth phase melanomas triggers migration, protease activation, and epithelial to mesenchymal transition (EMT), favouring progression from a radial to vertical growth phase phenotype [28]. It is also detected in subsets of childhood acute lymphoblastic and acute myeloid leukaemia where is associated with a worse prognosis in patients harbouring 11q23 translocations [128]. Aberrant CSPG4/NG2 expression has recently been found in renal and pancreatic cell carcinomas, chondrosarcomas, osteosarcomas, triple-negative breast cancer and squamous carcinomas of the head and neck [5, 51].

5.5 CSPG4/NG2 as a Therapeutic Target

5.5.1 CSPG4/NG2 in the Treatment of Gliomas

In gliomas, CSPG4/NG2 plays a central role in tumour cell proliferation through PDGFRα and FGF-2. Its aberrant overexpression in 50–67% of GBs, together with PDGFRα and Olig2, supports its use as a potential therapeutic target [7]. Consequently, the possibility of exploiting the theranostic properties of CSPG4/NG2 in gliomas and other tumour types has aroused considerable interest [5, 10].

CSPG4/NG2 overexpression is also positively associated with multidrug resistance, which is mediated by the increased activation of α3β1 integrin, PI3K/Akt signalling and their downstream targets, promoting cell survival [92, 129].

CSPG4/NG2 knockdown with shRNAs incorporated into lentiviral vectors attenuates β1 integrin signalling and has potent antitumour effects by sensitising tumour cells to cytotoxic treatment in vitro and in vivo [129].

In xenografts of the U87-MG GB cell line in athymic nude mice, chemoimmunoconjugates of

anti-CSPG4/NG2 mAb9.2.27 and vinblastine lead to long-term growth suppression [114]. A similar effect on tumour growth and angiogenesis has also been obtained in xenografts of GB-derived cell lines overexpressing CSPG4/NG2 by means of the intra-cerebral delivery of lentivirally encoded shRNAs [9].

Targeting CSPG4/NG2 with mAb9.2.27 and activated natural killer cells inhibits tumour growth and improves the survival of GB-bearing animals by favouring a pro-inflammatory TME [11, 12], as in a rat model of GB [13]. In comparison with single epitope targeting, a significant reduction in GB cell viability has been successfully obtained using a Mab-Zap saporin immunotoxin system to ablate CSPG4/NG2 and GD3(A), a ganglioside expressed by developing migratory glia [14].

The Cre-lox method for the cell type-specific ablation of CSPG4/NG2 [26] impairs tumour vascularisation in intracranial implantations of B16F10 melanoma cells in mice as a result of the loss of the CSPG4/NG2-mediated activation of β1 integrin signalling in pericytes [130].

5.5.2 CSPG4/NG2 as a Target for Immunotherapy

Given its overexpression in tumour cells, CSPG4/NG2 is an attractive candidate for antibody-based therapeutic approaches to solid tumours, including the use of specific anti-CSPG4/NG2 antibodies and immuno-based therapies (e.g. chimeric antigen receptor T [*CAR-T*] cell therapy) [15, 131–133].

As anti-CSPG4/NG2 mAbs inhibit tumour progression by blocking ligand access to extracellular CSPG4/NG2 binding sites, CSPG4/NG2-directed antibody conjugates are selectively internalised by CSPG4/NG2-expressing tumour cells as a result of endocytosis [132]. The upregulation of CSPG4/NG2 in tumour-associated pericytes means that this approach could contribute to tumour regression by inhibiting neo-angiogenesis in malignant brain tumours [15, 131].

The development of immunotherapy has led to significant progress in the treatment of indolent and metastatic tumours. By means of genetic engineering technologies, T lymphocytes can be redirected to recognise and target a wide variety of tumour-associated antigens (TAAs) through the expression of CAR-Ts. CARs are hybrid proteins in which the binding moiety derived from a mAb is fused with a signalling molecule of the CD3/T cell receptor complex and co-stimulatory endodomains. In order to overcome the need for T cells to recognise the TAAs presented by the major histocompatibility complex (MHC), CAR-T cells are genetically modified to express a chimeric T cell receptor that recognises the antigen of interest and redirects cytotoxic T cells to tumour cells. When inserted into T cells, CARs confer MHC-independent cytotoxic activity and promote T cell proliferation, activation and persistence both in vivo and in vitro [134].

Clinical trials of CAR-transduced peripheral blood lymphocytes have successfully led to the remission of both solid and haematological malignancies. In particular, redirected T cells expressing a CSPG4/NG2-specific CAR may be a promising new means of targeting a wide range of indolent solid tumours including GB [131, 135]. In a preclinical study, anti-CSPG4/NG2 CAR-T cells successfully induced growth arrest in GB-derived NS and glioma xenograft models, without any signs of immune evasion [16]. Remarkably, anti-CSPG4/NG2 CAR-T therapy is also effective in GB-derived NS expressing moderate to low CSPG4/NG2 levels, an effect mediated by the in vivo upregulation of CSPG4/NG2 under conditions of inflammation and hypoxia [136]. Inflammatory cytokines, including interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α), induce CSPG4/NG2 expression on tumour cells and microglia/macrophages, favouring their extravasation into the tumour mass. In their turn, antigen-activated CAR-T cells produce TNF-α in the glioma TME and induce CSPG4/NG2 expression even in the CSPG4/NG2– fraction of GB cells [137]. Constitutive and TNF-α-inducible CSPG4/NG2 expression may reduce the risk of tumour cell

escape when targeted antigens are heterogeneously expressed on tumour cells. At the same time, it keeps the tumour susceptible to targeted therapy, including CAR-T cell or mAb therapy (mAb9.2.27) [15, 138].

Two particular phenotypic features of GB must be considered when using this therapeutic strategy: (i) the presence of circumscribed necrosis with GSCs/progenitors spared from the advancing necrotic process or induced by TME/necrosis [139]; (ii) OPC and macrophage/microglia proliferation at the tumour border favours the tumour cell acquisition of a stem cell profile and chemoresistance. Known as the "border niche", this sanctuary may be very important when considering therapeutic strategies [122, 124–126].

5.6 Conclusions

Over the last few decades, a number of studies have shown that CSPG4/NG2 is a key factor in the development of the CNS and neuronal function, as well as in experimental and human glial tumours. Its role in CNS development, neo-angiogenesis and gliomagenesis emphasises its potential as therapeutic target, and new insights suggest that this may contribute to the defeat of glial neoplasia in the near future.

Studies of the function of CSPG4/NG2 have proved to be very useful in furthering our understanding of CNS biology, mainly because of its involvement in the cytogenesis of neurons or glia cells and normal neo-vasculature. They have also been important in clarifying the origin of gliomas and improving prognostic predictions. The dynamic expression of CSPG4/NG2 during cytogenesis could be used to establish the timing of the malignant transformation of gliomas and its significance in each molecular subtype. It would also be very interesting to verify its possible relationships with stemness or differentiation markers.

The increased expression of CSPG4/NG2 in many malignancies has led to it being considered a prototype oncoantigen and a putative candidate for targeted treatments. Clinical studies of malignant melanomas and triple-negative breast cancer have shown that it is a promising target for CAR-T cell therapy, and immunotherapeutic anti-CSPG4/NG2 CAR-T cell therapy may also be effective for malignant brain tumours because of the possibility of overcoming tumour escape and intra-tumour heterogeneity.

Increasing our knowledge of the role of CSPG4/NG2 in oncogenic signalling and TME interactions in GB (including the switch from proliferative to invasive programmes) will be critical objectives of investigations aimed at discovering all of the possible theranostic implications of this macromolecule.FundingThis work was supported by Fondazione Compagnia di San Paolo (Turin, Italy) (Grant No. 2016.AAI2705.U3302) and Fondazione Edo ed Elvo Tempia Valenta – ONLUS (Biella, Italy).

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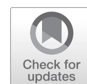

6 Lipoproteins and the Tumor Microenvironment

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Abstract

The tumor microenvironment (TME) plays a key role in enhancing the growth of malignant tumors and thus contributing to "aggressive phenotypes," supporting sustained tumor growth and metastasis. The precise interplay between the numerous components of the TME that contribute to the emergence of these aggressive phenotypes is yet to be elucidated and currently under intense investigation. The purpose of this article is to identify specific role(s) for lipoproteins as part of these processes that facilitate (or oppose) malignant growth as they interact with specific components of the TME during tumor development and treatment. Because of the scarcity of literature reports regarding the interaction of lipoproteins with the components of the tumor microenvironment, we were compelled to explore topics that were only tangentially related to this topic, to ensure that we have not missed any important concepts.

Keywords

Tumor environment · Lipoproteins Endothelial cells · Pericytes · Macrophages Exosomes · Fibroblasts · Neutrophils Microvasculature · Macrophage polarization Tumor aggressiveness · Metastasis · Immune surveillance · Adipose cells · High-density lipoproteins

6.1 Lipoproteins

Lipoproteins are pseudo-micellar structures, designed to transport essential water-insoluble molecules of dietary or metabolic origin: triacylglycerols for energy utilization and storage and cholesterol (Fig. 6.1) for membrane and hormone

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biogenesis and for bile acid synthesis. Lipoproteins, especially high-density lipoproteins (HDLs), also transport a broad range of substances, including drugs and nucleic acids [1], that could contribute to carcinogenesis, metastasis, or other (therapeutic) processes opposing these phenomena. As a result, lipoprotein-type delivery vehicles have been investigated as anti-cancer agents for decades [2], primarily because of their ability to specifically target malignant cells and tumors via receptor-mediated interactions [3]. The special affinity of the apolipoprotein components of the lipoprotein complexes or their mimetic surrogates [4] for specific receptors is likely to extend to macrophage receptors or to other surface antigens found in the TME. Consequently, lipoprotein-type carriers could become important therapeutic tools in the treatment of especially aggressive cancers. In addition, the study of the interaction between lipoproteins and components of the TME could reveal important information, inducing new therapeutic approaches for difficult to treat malignant tumors. Currently, information available regarding the role that lipoproteins play in cancer development, diagnostics, and therapeutics by interacting with components of the TME is very limited the scientific/medical literature. This review is an attempt to focus attention on these interactions that will likely play a significant role in the design and development of novel diagnostic and treatment approaches in oncology.

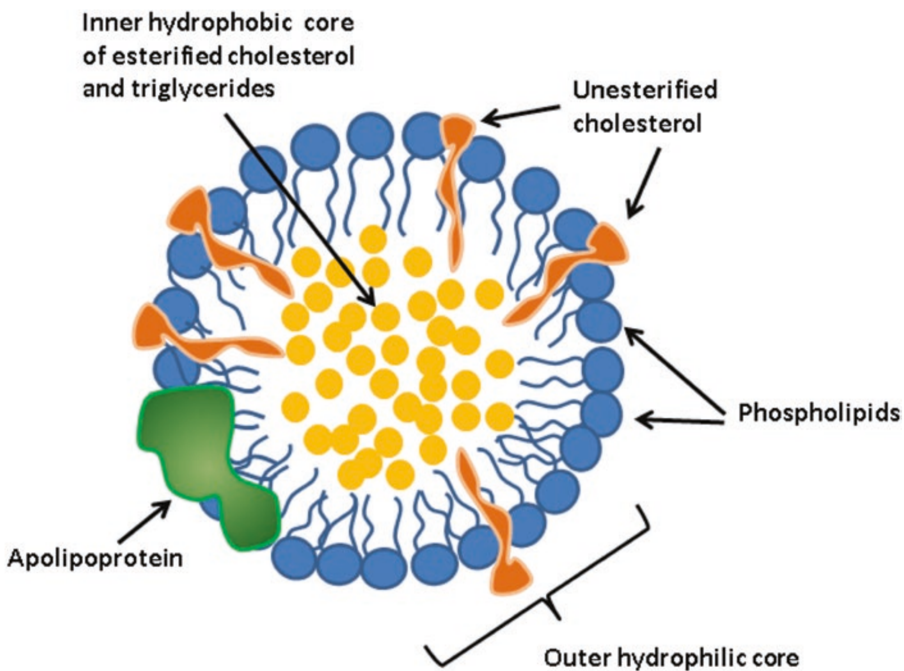

Fig. 6.1 Schematic structure of a generic plasma lipoprotein (From: *Bioscience notes*. Online biological notes for students http://www.biosciencenotes.com/lipoproteins-introduction-structure-and-function/)

6.2 Contributions of Specific Components of the TME to Tumorigenesis and Metastasis

Cancer has been recognized as a highly heterogeneous disease, involving a multicomponent tumor environment that is comprised a variety of resident and infiltrating host cells including myofibroblasts, neuroendocrine cells, adipose tissue, immune inflammatory cells, blood, and other vascular lymphoid networks [6], in addition to tumor cells (Fig. 6.2).

The interplay between these cells provides growth signals, secretory factors, metabolites, and extracellular matrix proteins and thus creates a favorable environment, collectively known as the stroma, for tumor growth and metastasis. A deeper understanding of the interactions within the TME has now revealed additional details, including key secretions originating from tumor cells that have the ability to alter the phenotypes

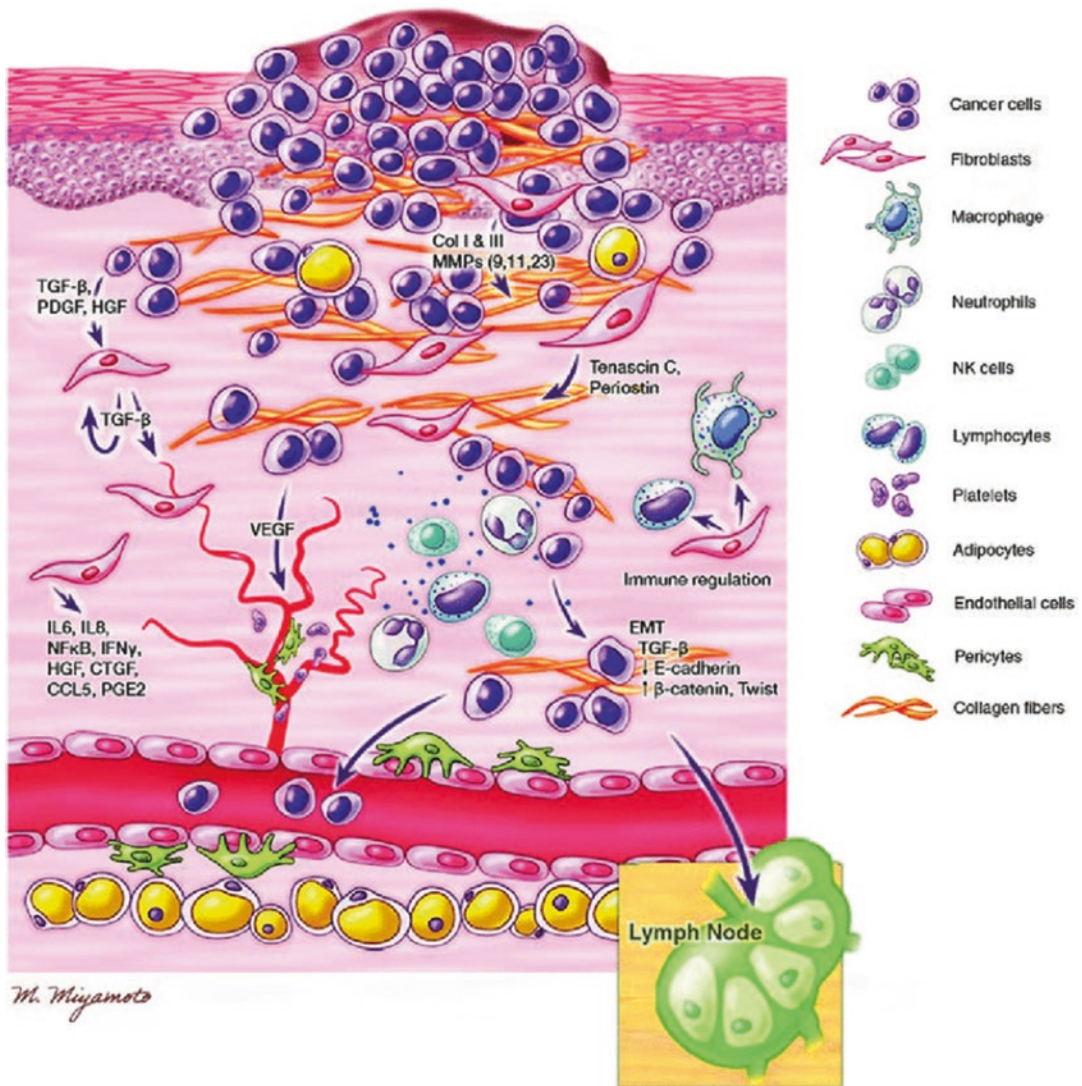

Fig. 6.2 Ingredients frequently found to contribute to interactions within the tumor environment (From: Foster et al. [5])

of immune cells and thereby suppressing the immune system and thus promote tumor growth and metastatic processes [7, 8]. It has recently been shown that tumor diversity is enhanced via the varying components of their environment, including stromal cell proportions or activation states. In response to evolving environmental conditions and oncogenic signals from growing tumors, the TME continually changes over the course of cancer progression, underscoring the importance of the TME and the understanding of how tumor cells influence the assembly of their environment [9]. Based on the current findings of Wang et al. [6], the TME is highly influenced by 10 important characteristics including:

(a) Uncontrolled multiplication
(b) Escape from growth suppressors
(c) Promotion of invasion and metastasis
(d) Resisting apoptosis
(e) Stimulating angiogenesis
(f) Maintaining proliferative signaling
(g) Elimination of cell energy limitation
(h) Evading immune destruction
(i) Genome instability and mutation
(j) Tumor-enhanced inflammation

Besides these factors that contribute to an optimal environment for tumor growth, other conditions, including acidity (low pH) in the TME, may also be important contributors to angiogenesis and metastasis [10]. Additionally, a bidirectional communication between the tumor cells and the microenvironment is another critical factor that influences the initiation and progression of malignancy and eventually patient prognosis [11]. Hence, understanding the role of the tumor microenvironment and monitoring its changes via molecular and cellular profiles during tumor progression could be vital for identifying cellular or protein targets for cancer prevention and therapy [12, 13].

As described above, tumorigenesis is a complex three stage and dynamic process: initiation, progression, and metastasis. The tumor microenvironment (TME) consisting of stromal cells and extracellular matrix (ECM) has been shown to be modified (educated) by tumor secretions, leading to a synergistic relationship between the constituents of the TME and the tumor itself. Consequently, the steps involved in tumorigenesis are determined/influenced by the composition and functional activity of the TME, especially via immunogenic and non-immune cells. In the following sections, the opportunities for novel diagnostic and treatment approaches of malignant tumors will be discussed with a special focus on the potential interactions that may exist between lipoproteins and the TME.

6.2.1 Contributions of Lipids/Lipoproteins to Tumor Acidosis in the TME

Although a complex milieu in TME triggers oncogenic events that drive malignant developments; stress factors such as hypoxia and acidosis also have a key role in early tumor development. Many of the early metastatic events are supported by a "reversed pH gradient," representing fluctuations between intracellular and extracellular pH [14, 15]. Lipid/lipoprotein metabolism has been linked to changes in lipid availability, thus impacting the metastatic potential of malignant cells and tumors. Lipids accumulate in special cytoplasmic organelles, termed "lipid droplets" (LDs) or adiposomes, composed primarily of neutral lipids (triacylglycerols and cholesteryl esters) that primarily originate from lipoproteins [16, 17]. Lipid droplets also serve as a reservoir for cholesterol and acylglycerols for membrane biogenesis and energy-yielding metabolites [18, 19]. Several studies have shown abnormal accumulation of LDs in malignant tumors [17, 20–23]. Koizume et al. [24] reviewed the production and functions of LDs in cancer cells in relation to the cellular environment, including tissue oxygenation status and metabolic activity. These findings contributed to the current understanding of how cancer cells adapt to diverse tumor environments to promote their survival via accelerating their lipid metabolism. In another study, LD accumulation has been demonstrated to be affected by environmental stresses, including acidosis, hypoxia, as well as chemoresistance [22] in addition to de novo lipogenesis and extracel-

lular lipid uptake [25, 26]. In the TME, LDs may also play an important role in ER homeostasis [27] and as ROS scavengers [28, 29].

6.2.2 Cancer-Activated Fibroblasts (CAFs)

Fibroblasts are cells that regulate the structure and function of healthy tissues through the formation of the extracellular matrix (ECM). Myofibroblasts, which are activated fibroblasts, are transiently present at the site of tissue injury, involved in wound repair, and progressively disappear via an apoptotic mechanism. However, during cancerous tissue fibrosis, myofibroblasts, called cancer-associated fibroblasts (CAF), are permanently activated and are essential for initiating and promoting tumor growth [30–32]. CAFs are one of the most dominant components in the tumor stroma [33] and have been identified to exert a significant control on the rate and the progression of tumor growth by remodeling ECM proteins, secreting growth factors and immunosuppressive cytokines, recruiting inflammatory cells, inducing angiogenesis, and enhancing cancer cell proliferation via mesenchymal-epithelial cell interactions.

In addition, there are several subsets of CAFs that have been known to exhibit specialized functions in the carcinogenesis. Investigation of proliferative ability of CAFs has revealed that α-smooth muscle actin (α-SMA)-expressing CAFs (also known as myofibroblasts) promote the proliferation of cancer stem cells by utilizing the interaction between the chemokine receptor and its ligands, CXCR4 and CXCL12 [34]. A specific subset of CAFs in pancreatic cancer (that express α-SMA, vimentin, and glial fibrillary acidic protein) secrete macrophage colony-stimulating factor (M-CSF), interleukin 6 (IL-6), and CC-chemokine ligand 2 (CCL2) and thus promote monocyte recruitment and additionally macrophage differentiation and polarization to the M2 (tumor-promoting) tumor-associated macrophages (TAM) phenotype [35, 36]. The α-SMA-secreting CAFs also secrete cytokines (IL-6, IL-8, TGF-, and IL-10) to recruit monocytes and to promote their differentiation toward the M2 phenotype. Interestingly, M2 macrophages further activate the CAFs leading to further (self-catalytic) tumor progression [37, 38]. The expression of M2 macrophage markers (CD163 and DC-SIGN) and CAF markers (α-SMA, S100A4, and FAP (fibroblast-activating protein)) has been predictors of poor clinical outcomes in squamous cell carcinoma and colorectal cancer patients [39, 40].

A potential therapeutic approach involving lipoprotein transport that is discussed in more detail below, under the topic dealing with TAMs, could potentially also be useful against CAFs via limiting the polarization of TAMs toward the tumor-promoting M2 phenotype. Takai et al. [41] reported substantial reduction of tumor growth in triple-negative breast cancer xenografts of patient-derived tumors in mice using pirfenidone, a lipophilic anti-CAF agent. Utilizing lipoprotein-based drug delivery that is exceptionally suitable for transporting lipophilic drugs, in addition to the selective targeting potential via the scavenger receptor class B type 1 (SR-B1) receptor [16], could significantly enhance this therapeutic approach.

6.2.3 Neuroendocrine Cells

Neuroendocrine tumors (NETs) are heterogeneous malignancies that arise in specialized neuroendocrine (NE) cells. These cells are part of the diffuse neuroendocrine system which exhibits a combination of both neuronal and endocrine features [42]. In general, NE cells respond to neurological signals by secreting and releasing hormones and peptides into the bloodstream, inducing physiological and local regulatory functions in specific organ sites, including the regulation of appetite, body temperature, pH, etc. [43]. NETs may be functioning or nonfunctioning tumors as exemplified by pancreatic tumors that produce a variety of peptide hormones including insulin, glucagon, and gastrin, whereas metastatic NETs have been found to secrete serotonin and other vasoactive substances [44].

Recently, tumor neoangiogenesis has been observed to play the key role in NET progression. The TME makes an important contribution to the progression of NETs and to the pathogenesis of fibrotic complications of carcinoid heart disease and mesenteric desmoplasia. NE cells also play an important role in the development of NE tumors. Accordingly, investigation of NE cells in prostate carcinoma has revealed that their secretory products enhance the proliferation of prostate tumors (by inhibiting apoptosis and stimulating neoangiogenesis) and thus have been linked to tumor aggressiveness [45]. Although rare, the incidence of NETs is relatively high in the gastroenteropancreatic (GEP) tract and in the bronchopulmonary (BP) tract [44]. Recent studies show that both gastrointestinal NETs and pancreatic NETs respond to everolimus and temozolomide [46]. We have recently shown that both of these agents were effective in suppressing the growth of glioblastoma cells, especially when delivered via high-density lipoprotein-type drug transport system to SR-B1 overexpressing cells [47]. A similar drug delivery approach might be effective against NET cells if the reconstituted high-density lipoprotein nanoparticles (rHDLs) are augmented by conjugation of somatostatin mimetic peptides, targeting NET surface receptors [48].

6.2.4 Neutrophils

Originating from the bone marrow, neutrophils are short-lived cells and represent the most abundant circulating leukocytes, and along with macrophages, they make up the first line of defense of the innate immune system. Recruited from the circulation by cytokines and chemokines, neutrophils infiltrate tissues and work to contain infection. Phagocytosis and subsequent degradation of the engulfed microbes via proteases and respiratory burst constitute a major mechanism of the antimicrobial activity of neutrophils [49, 50]. Another method that neutrophils utilize to target microbes is degranulation in the extracellular space. Diverse types of granules have been identified in neutrophils, including azurophil granules that take up the azure A dye and store the microbicidal azurocidin and myeloperoxidase (MPO) [51]. Specific granules contain the non-heme iron-binding protein, lactotransferrin, that contributes to the antimicrobial effects [52–55]. Anti-inflammatory effects have also been reported for lactotransferrin while studying microbial and auto-immune diseases [56–58]. Besides actively fighting of microbes, neutrophils can also modulate the function of other immune cells including macrophages, dendritic cells, monocytes, natural killer (NK) cells, T cells, and B cells via direct interaction or the release of cytokines [59].

Meta-analyses across several cancer types and stages show that high neutrophil-to-lymphocyte ratio in the blood and high intratumoral neutrophil infiltration correlate with adverse outcomes [60–62]. Despite their prognostic value, controversy surrounds the role of neutrophils in the TME, as they can release both cytotoxic, proangiogenic, and pro-metastatic factors [63]. For example, neutrophils from healthy donors exert cytolytic activity on cancer cells via the production of reactive oxygen species [64]. Paradoxically, tumor-associated neutrophils (TANs) represent a major source of metalloproteinase-9 (MMP-9) that can break down the extracellular matrix (ECM), inducing the release of VEGF from the ECM [65–67]. Studies in mice advanced the identification of two subsets of TANs: the pro-inflammatory, anti-tumoral N1, and the immunosuppressive, pro-tumoral N2 TANs with the N2 phenotype induced by TGF-β in the TME [68]. In line with these observations, high N2-to-N1 ratio positively correlated with lung cancer aggressiveness [69]. Moreover, Eruslanov et al. reported that in early-stage lung cancer, “activated” (N1-like) TANs are able to stimulate T cell proliferation and activation [70]. Besides the N1 versus N2 functional model, other descriptions of pro-tumoral versus anti-tumoral neutrophils have been reported including the low-density neutrophils versus the normal-density neutrophils and the pro-tumoral immature granulocytic myeloid-derived suppressor cells [71].

Lipoproteins and TANs Earlier studies of vascular inflammatory diseases showed that lipoproteins do affect the function of neutrophils. In vitro treatment of neutrophils with Apo A-I and HDL or treating mice with Apo A-I and HDL or pre-treating patients with peripheral vascular disease with reconstituted high-density lipoproteins resulted in decreased neutrophil activation, adhesion, and migration. This dampening of neutrophil activation was attributed to the interaction of Apo A-I and HDL, respectively, with the ABCA1 and SR-B1 receptors [72]. Divergent effects on neutrophils have been described on native and oxidized LDL. In a model of chronic hypercholesterolemia with an elevated level of oxidized LDL, neutrophil calcium influx and chemotaxis were impaired, whereas expression of CD11b-surrogate for activation state of neutrophils was not altered. Although both native LDL and oxidized LDL significantly induced the expression of the monocyte chemoattractant protein 1, treatment with native LDL increased CD11b expression, chemotaxis, and calcium flux in neutrophils, whereas oxidized LDL abrogated these effects and promoted apoptosis of neutrophils [73]. Obama et al. showed that oxidized LDL, but not native LDL, enhanced phorbol 12-myristate 13-acetate-induced NET formation and myeloperoxidase production in neutrophils [74]. Because oxidized LDL is a contributor of carcinogenesis ([75]; [76]), investigation of the interaction between lipoproteins and infiltrating neutrophils and how these interactions may modulate the function of neutrophils in the TME is likely to lead to important advances and perhaps novel diagnostic and therapeutic approaches.

6.2.5 Endothelial Cells in the TME

Cancer cells require a constant, vigorous supply of nutrients and oxygen to facilitate the rapid growth and the homeostasis of the TME. Angiogenesis, including the growth of new capillaries, is also stimulated by oxygen and nutrients. Apparently, during hypoxia or oxidative stress, tumor cells are under stress to secrete proangiogenic ligands that induce the endothelial cells to initiate the process of angiogenesis or neovascularization. The tumor-associated endothelial cells (TNECs) play two key roles in the TME:

1. TNECs enable the development of new blood vessels from the existing blood vessels or by recruiting bone marrow-derived progenitor endothelial cells.
2. TNECs mechanistically control the recruitment of leukocytes, tumor cell behavior, and consequently even metastasis (Chouaib S) as they are crucial components of the interface between the circulating blood cells, tumor cells, and the extracellular matrix (ECM). Tumorigenesis may be impacted differently under hypoxia or inflammation [77]. For instance, HDL has been found to augment angiogenesis during hypoxia, while it has been reported to inhibit angiogenesis under inflammatory conditions.

The precise mechanism involving the role of HDL and endothelial cells in the TME is still under investigation as Yu et al. [78] reported that endothelial lipase (LIPG) may be involved in HDL- induced angiogenesis. LIPG is a phospholipase, secreted by endothelial cells, and plays a vital role in lipoprotein metabolism, specifically in HDL metabolism. Accordingly, several studies attributed the effect of HDL on angiogenesis to its association with its active lipid ligand, sphingosine phosphate [79, 80]. HDL contains sphingosine-1-phosphate (S1P) that besides triggering a vascular response is also a substrate for S1P receptor (S1PR) on endothelial cells [81]. Subsequently, studies conducted by Tatematsu et al. [82] invoked a possible mechanism for LIPG in tumor angiogenesis. Tatematsu's observations indicated that LIPG-mediated hydrolysis of HDL releases and activates S1P which upon binding to the S1PR promotes the phosphorylation of protein kinase B and endothelial nitric oxide synthase [82]. Consequently, this leads to the migration of endothelial cells and hence angiogenesis. Besides playing a role in angiogenesis, LIPG has also been observed to contribute to cell growth, cell proliferation, and some of

the crucial events in cancer progression [83]. Hence, LIPG could be explored as a potential target for cancer therapy. Intriguingly, several studies have also identified role(s) for apolipoprotein A-I (Apo A-I), the primary protein component of HDL, and its mimetic peptides in angiogenesis. Studies conducted in a mouse model of ovarian cancer indicated that the mice expressing the Apo A-I transgene had decreased tumor development and increased survival compared to the wild type mouse [84]. In another similar study, the effect of Apo A-I in tumorigenesis was investigated by analyzing the tumor-associated blood vessels in Apo A-I transgenic and knockout mice. The results indicated a significant decrease in the number of tumor blood vessels in addition to the reduction in vessel length, size, area, and density in the Apo A-I transgenic mice compared to the Apo A-I knockout mice [85].

Regarding the contribution of Apo A-I mimetic peptides to angiogenesis, subcutaneous or oral injection of mimetic peptide L-4F in CT26 mouse colon adenocarcinoma model showed a reduction in the proliferation and the viability of cancer cells. Subsequently, the peptide also decreased the tumor burden and the neovessel expression in tumorigenic BALB/c mice [86]. Similarly, investigation of the anti-tumorigenic effects of another Apo A-I mimetic peptide, L-5F, also revealed that the inhibition of vascular endothelial growth factor (VEGF)/basic fibroblast growth factor (bFGF)-mediated angiogenesis was partially responsible for the inhibition of tumorigenesis. In vitro studies showed that L-5F peptide inhibited the proliferation, invasion, migration, and the tube formation of endocytic cells by suppressing the VEGF/bFGF pathways [87]. Additionally, in vivo studies also indicated that the animals that received the peptide showed a decrease in the quantity and the size of the vessels in the tumor compared to the untreated group. Although several studies indicate that the anti-tumorigenic properties of HDL might occur via inhibition of angiogenesis, the contrasting characteristics of HDL with regard to hypoxia and inflammation suggest that its role in angiogenesis might be dependent on the type of cancer [77].

6.2.6 Stroma and Endothelial Cells

As increasing amounts of evidence indicate the presence of crosstalk between fibroblasts and NETs in the TME. Both in vitro and in vivo experiments conducted to analyze the effect of CAFs on NETs indicate that CAFs could promote the growth of NETs [44, 88]. However, the proliferative ability of NET cells has been linked only to the α-SMA^{+} myofibroblasts and not to resting fibroblasts. Additionally, studies by Bowden et al. [89] indicated that IL-6, VEGF, and monocyte chemoattractant protein 1 might play a role in stimulating NET cell proliferation based on comparing the secretomes of CAFs and normal human fibroblasts.

NETs are one of the most highly vascularized cancers, as they overexpress a large number of proangiogenic factors, including VEGF, FGF, PDGF, semaphorins, and angiopoietins [90]. Consequently, NETs have a high intratumoral density, almost tenfold higher than other carcinomas [91]. Interestingly, the intratumoral microvascular density is higher in low-grade pNET tumors than in high-grade tumors, and it is also associated with improved prognosis and longer survival [92, 93]. Among the angiogenic factors expressed in pNETS, VEGF is the most effective promoting angiogenesis, as it is highly expressed in almost 80% of the NETS. Additionally, tumor expression of VEGF is higher in well-differentiated tumors compared to the poorly differentiated ones. VEGF and angiopoietins have been described as major contributors to NET progression, in agreement with the Durkin et al. [94] who found that angiopoietin is significantly upregulated in pNETs. Furthermore, in vivo studies conducted by Rigamonti et al. [95] have shown that angiopoietin can increase the microvascular density of pNETs.

Regarding the importance of the penetration of the endothelial and stromal barrier of the TME, Wilhelm et al. [96] conducted groundbreaking studies, assessing the effectiveness of the delivery of anti-cancer agents to tumors and tumor cells via nanocarriers. After examining the findings from 232 datasets in 117 carefully selected

studies, they found that on the average, only 0.7% of the injected dose of nanoparticles actually reached the tumor. Follow-up studies [97] were even more alarming. These investigators specifically investigated the efficiency of tumor-targeted nanoparticles and found that only 0.0014% of the injected drug load actually reached the cancer cells within the tumor. The rest of the payload was apparently trapped in the TME. These findings brought into focus the significance of the physical/chemical and biological properties of the respective nanoparticles that have been used to deliver anti-cancer agents. In our hands, the reconstituted high-density lipoprotein nanoparticles (rHDL NPs) are highly efficient in eliciting nearly a 90% ovarian tumor suppression upon injecting only a small (5 μg) siRNA dosage [98]. Perhaps the extremely small size of the nanoparticles (<20 nm in diameter) allowed the efficient penetration of the tumor via the available openings in the tumor environment, in addition to the targeted delivery of the nano-payload via the SR-B1 receptor.

6.2.7 Blood and Lymphatic Vascular Networks (BLVN)

A major function of the blood and lymphatic vascular network in the TME is to supply oxygen and nutrients for the survival and growth of the specific components, including cancer cells, to regulate interstitial pressure in tissues and organs, as well as to remove carbon dioxide and metabolic wastes from the surrounding milieu. Unlike the healthy vasculature, tumor vessels exhibit highly abnormal structural and functional characteristics [99–101], including irregular architecture with a highly dysfunctional and leaky endothelial cell (EC) layer. These and other unique features of the TME interfere with the anti-tumor immune defenses while enhancing the tumor's aggressiveness, facilitated by the hypoxic and acidic ambience and enhanced angiogenesis.

One of the primary functions of the BLVNs is to assist tumor cells to escape immune surveillance that has been suggested to operate via the programmed death-1 (PD-1) T cell co-receptor and its ligands [102]. This pathway has also been identified as a potential therapeutic target via PD-1 blockade. Lymph nodes provide a favorable microenvironment for the seeding and proliferation of cancer cells, due to local secretions that facilitate tumor growth [103, 104]. In addition, vascular endothelial growth factor (VEGF)-targeted therapies were initially developed to inhibit new blood vessel growth and thus starve the tumors of necessary oxygen and nutrient supply. When administered either as a single agent or in combination with chemotherapy, these agents have been shown to benefit patients with advanced-stage malignancies [105–107]. Regarding interactions with lipoproteins in either in the blood or via lymphatic networks, dietary, metabolic, and pathological changes could lead to marked alterations in the lipid/lipoprotein profile of cancer patients. For example, decreased blood cholesterol, especially HDL cholesterol, levels have frequently been observed in cancer patients [108, 109]. These and other changes in the patient's lipid profile [110] could substantially change the function of TME ingredients, upon interaction with the specific lipoproteins of altered composition. Finally, lipoproteins could serve as drug carriers and thus alter the impact of the chemotherapeutic agent administered [111, 112] for treatment. This is a continued key consideration when lipophilic anti-cancer agents are involved in the treatment strategy of a particular malignancy.

6.2.8 Adipose Cells

White adipose tissue cells (adipocytes) reside in the TME [113] as [63, 114] suggested that they are one of the primary stromal cells that play an active role in the TME. Cancer-associated adipose cells (CAACs) are frequently found adjacent to cancer cells and have the ability to communicate with tumor cells via secretion of cytokines, chemokines, and hormone-like factors [113, 115, 116]. A crosstalk between adipocytes and cancer cells thus may result in functional and phenotypic changes in

both cell types, which can further promote tumor progression. Roberts et al. [117] and Naugler et al. [118] have found that adipose tissue cells can induce a highly pro-inflammatory environment via contributing to tissue hypoxia that in turn enhances TME tumorigenesis. In addition, increased accumulation of pre-adipocytes has been observed in obese patients, with an enhanced level of macrophages and monocytes that secrete pro-tumor substances and thus promote tumor growth and metastasis [119, 120]. Re-programming of adipose tissue along with recruitment of progenitor mesenchymal stromal cells (MSCs) under hypoxic and inflammatory conditions has been shown to mediate cancer progression [120–122]. The impact of lipoproteins on adipose cells has been investigated for several decades [123–125]. More recently, a model emerged that should allow the investigation of changes in lipoprotein metabolism on adipocytes in the tumor environment [126].

6.2.9 Pericytes

Originally described as essential components of the microvasculature [127], more recently pericytes have been isolated from a number of different tissues [128] where they fulfill important physiological roles, by themselves or via interacting with other types of cells [129]. Regarding interactions within the TME, pericytes have been mentioned as contributors to the buildup of the microvasculature in ovarian cancer models [130] and during breast and prostate carcinogenesis [131]. Perhaps most of the tumor-promoting potential of pericytes is expressed in the vicinity of brain lesions [132, 133] where pericyte targeting as a therapeutic approach has also met with some success [132].

Regarding interactions with lipoproteins, pericytes were shown to clear amyloid-β42 aggregates using an apolipoprotein E isoform-specific mechanism, via the LRP1 receptor [134]. Within the TME, interactions with apoE isoforms while possible, especially in the brain, so far have not been documented.

6.2.10 Extracellular Vesicles

Extracellular vesicles (EVs) are membrane-bound structures that serve as transporters and intercellular communicators that play an important role in the TME. EVs are nanosized, consist of a lipid bilayer, and are classified as microvesicles (MVs), exosomes, oncosomes, and apoptotic bodies, depending on their biogenesis. EV subtypes are distinguished by their targeting ability to recipient cells to mediate specific biological functions. EVs are secreted via different mechanisms and interact with target cells using different uptake mechanisms, are produced by most tissue types including tumors, and found in milk urine, seminal plasma, and blood and lymphatic vessels [135]. The components of EVs include lipids, nucleic acids, metabolites, and proteins, primarily derived from donor cells [136].

Current studies reveal important similarities between EVs and lipoproteins as both carry and deliver miRNAs to target cells and both were shown to carry most apolipoproteins. The size and density of EVs closely overlaps those of lipoproteins, consequently, isolation and purification of either entity from blood sample results in co-purification with the other [137] [138–140]. Common structural and functional characteristics exhibited by EVs and lipoproteins could be significant in assessing their roles in the TME. EVs also share similar features with viral particles, as carriers of RNA and proteins [141]. EVs and lipoprotein mimetic particles are currently being developed as drug delivery vehicles due to their stability, half-life, and targeting capabilities [142–144]. Interestingly, lipoprotein-like particles have been shown to transfer lipids to EVs intracellularly following SR-B1-mediated uptake, further highlighting the possible interplay between the two particle types.

Depending on their origin and target cell destination, EV internalization may occur via multiple processes, including micropinocytosis, phagocytosis, direct membrane fusion, or receptor-mediated endocytosis. While the proteome of EVs has been mapped, the key challenges are in identifying potential specific binding ligands and alternative uptake receptors that facilitate cargo

transport via EVs in TME. Considering that hypoxia and acidosis are general features of aggressive tumors, the role(s) of circulating EVs as dynamic biomarkers is likely to evolve in the near future.

6.2.11 Tumor-Associated Macrophages

Macrophages, as cellular components of the innate immune system, are characterized by heterogeneity of their surface marker expression and secretory profile [145–147]. All vertebrate tissues harbor macrophages which are participating in tissue homeostasis, including organ development, mediation of inflammation, wound healing, and repair [147, 148]. Although macrophages may exhibit some tissue-dependent functions, their major physiological role lies in the detection, phagocytosis, and clearance of "non-self" objects, apoptotic cells, and other cellular debris and pathogens [145, 149, 150]. Thus, macrophages are considered to serve as the "first responders" of the immune system. In this defensive role, macrophages enhance the function of the immune system by producing chemokines and cytokines which mediate the recruitment and activation of monocytes and other immune cells from the blood to facilitate their transit to the tissue site that has been breached. Along with dendritic cells, macrophages bridge the primary immune response from the innate to the adaptive system by presenting antigens to T cells after engulfing foreign substances or organisms [151].

In contrast to their pro-inflammatory role, macrophages can dampen immune activation via the expression of immune checkpoint ligands and the production of immunosuppressive cytokines [152, 153]. Moreover, macrophages can promote tissue remodeling and repair at the wound site through the secretion of growth factors, matrix metalloproteinases, and the recruitment of tissue fibroblasts [154–156]. This dual role of macrophages in the modulation of inflammation is attributed to their ability to assume a spectrum of phenotypes, elicited by environmental triggers. Two major activation states of macrophages drive their respective functions: the classically activated macrophage (or M1-type macrophages) and the alternatively activated macrophages (or M2-type macrophages). Physiological conditions that require a pro-inflammatory response induce an M1 phenotype in macrophages, and the M2 phenotype emerges when immune suppression, tissue repair, and remodeling are needed [157, 158]. The paradigm of this dual role of macrophages has been key in understanding the contribution of tumor-associated macrophages (TAMs) to tumor progression. TAMs represent an important part of the immune landscape of the tumor microenvironment (TME). They are found in the stromal compartment of tumor and may contribute up to 50% of the tumor mass [159]. Although some TAMs may be classified as tissue-resident macrophages, the majority are derived from circulating monocytes which are continuously replenished through recruitment from the blood [160, 161].

6.2.11.1 Tumor-Associated Macrophages in the Tumor Microenvironment

Through the action of tumor-derived cytokines and chemokines such as interleukins (ILs), the C-C motif chemokine ligand 2 (CCL2), the CCL3, the C-X-C motif chemokine 12 (CXCL12), and the colony-stimulating factor 1 (CSF1), circulatory monocytes are recruited to the TME and differentiate to TAMs. In the TME, TAMs progressively take on an M2 phenotype which enables tumor progression and metastasis [160–163]. Factors contributing to the M2-like phenotype of TAMs include CSF1, the placental growth factor (PlGF), the vascular endothelial growth factor (VEGF), and the transforming growth factor beta (TGFβ) [164–166], as well as extracellular vesicles [167]. Some of these factors including VEGF and TGFβ may be subsequently released by TAMs to act in an autocrine or paracrine manner in the TME, reinforcing their M2-like phenotype and driving tumorigenesis and metastasis [166, 168, 169]. The hypoxic environment of the tumor is another factor which sustains the pro-tumoral

(M2) phenotype of TAMs. This environment stimulates the production of proangiogenic factors such as VEGF in the TME through the activation of the transcription factors hypoxia-inducible factor 1-alpha (HIF1α) and HIF2α in not only tumor cells but also in other cells, including TAMs, present in the TME [170, 171].

In several cancer types, the abundance of M2-like TAMs in the TME (especially CD163+ CD204+ TAMs) has been associated with poor patient prognosis [172–177]. This pro-tumoral effect of TAMs has been linked to their ability to mediate immunosuppression, extracellular matrix remodeling, and the epithelial-to-mesenchymal transition (EMT), to produce proangiogenic and lymphangiogenic factors and to help the establishment of pre-metastatic niches [33, 178–182]. In epithelial ovarian cancer, microRNAs carried in TAM-derived exosomes were reported to contribute to the high regulatory T cells (Treg)/T helper cell 17 (Th17) ratio and thus promoting an immunosuppressive environment in the TME [183]. In line with the immunosuppressive effect of TAMs, high TAM count correlated with high Treg count and with poor prognosis in non-muscle invasive bladder cancer when treated with Calmette-Guérin vaccine [184]. In addition, the infiltration of $CD8^+$, cytotoxic T cells into the TME is inhibited by TAMs in human lung squamous cell carcinoma [185]. The high expression of immune checkpoint components such as the signal-regulatory protein alpha (SIRPα) and the programmed cell death protein 1 (PD-1) or its ligand (PD-L1) on the surface of TAMs is a mediator of macrophages phagocytosing cancer cells and the recruiting and activation of cytotoxic immune cells [186–188]. Because TAM-mediated immune suppression allows cancer cells to thrive undetected and unimpeded by the immune system, several TAM-targeted cancer immunotherapy strategies have been developed to either inhibit circulatory monocyte recruitment, deplete TAMs, or repolarize TAMs from an M2 phenotype back to an M1 phenotype [160, 189].

6.2.11.2 Lipoproteins and Tumor-Associated Macrophages

It is well documented that low-density lipoproteins (LDL) are atherogenic once they become oxidized and are engulfed by macrophages. At the same time, high-density lipoprotein (HDL) is considered athero-protective by removing the excess cholesteryl ester from the macrophage foam cells, via reverse cholesterol transport (RCT), and promoting an anti-inflammatory response in arteries [190–192]. These effects are primarily mediated by the interaction of the two major structural protein components of HDL (apolipoprotein [Apo] A-I and Apo A-II) and receptors on target cells. While the interaction of Apo A-I with the ATP-binding cassette transporter A1 (ABCA1) or ABCG1 stimulates the translocation of lipids to the cell surface and promotes cholesterol efflux to nascent HDL, its interaction with the scavenger receptor type B class 1 (SR-B1) on hepatocytes promotes cholesterol influx from the mature HDLs [193–195]. The SR-B1 expressed on non-hepatic cells, including macrophages, has been shown to participate in both influx and efflux of cholesterol, depending on the need of the cell [196, 197]. Besides modulating lipid trafficking, receptors can also initiate signal transduction and thus may have a role in tumorigenesis [198].

Although lipid loading of macrophages via lipoproteins has been extensively studied in the context of the pathogenesis of atherosclerosis, less is known about the interaction of lipoproteins with TAMs and the impact of such interaction on the phenotype and function of TAMs in the TME. However, recent findings suggest that lipid efflux from or influx into TAMs mediated by lipoproteins can impact the inflammatory profile of TAMs. Already, intratumoral cholesterol accumulation has been demonstrated to enable tumor progression in several types of cancers due to the ability of cholesterol and its derivatives to influence cell signaling by serving as a ligand to cellular receptors and by stabilizing lipid rafts [199, 200]. Although fatty acid influx into macrophages and

subsequent fatty acid oxidation have been observed as contributors to TAMs' energy metabolism [201], it may not be required for the maintenance of the immunosuppressive M2 phenotype [202] while fatty acid synthesis has been observed with the M1 pro-inflammatory macrophage phenotype [203]. With macrophages having a high lipid uptake capability, it is reasonable to assume that the interaction of TAMs with lipoproteins could profoundly impact tumor progression. Meanwhile, several studies have explored the impact of lipoproteins on tumor progression in general. For example, Lu et al. [204] reported that breast cancer cells treated with very low-density lipoprotein (VLDL) and LDL upregulated proliferative, survival, angiogenesis, drug resistance, and metastatic pathways, whereas HDL-treated breast cancer cells did not. Accordingly, mice with LDL receptor (LDLR) silencing or LDLR knockout exhibited reduced tumor burden [205]. In esophageal squamous cell carcinoma, LDL treatment of cancer cells increased cell viability, and plasma LDL levels negatively correlated with patient overall survival [200].

While these findings underscore the tumor-promoting role of LDL perhaps associated with the intratumoral accumulation of cholesterol, they also suggest that the interaction of LDL with TAMs could alter their phenotype to an M2-like, pro-tumoral profile and that interaction of HDL with TAMs could yield an M1 phenotype with anti-tumoral effects. This paradigm may seem counter-intuitive as HDL mediates an anti-inflammatory state in blood vessels, while LDL tends to be pro-inflammatory. However, the interaction of oxidized LDL with the scavenger receptor CD36 and the platelet-activating factor receptor on bone marrow-derived macrophages resulted in a polarization toward the M2 phenotype [206]. Injection of Apo A-I and HDL in a murine model of melanoma resulted in tumor regression. This anti-tumoral effect was shown to be mediated by the infiltration of M1-type TAMs into the tumor which, in turn, promoted increased infiltration of the cytotoxic CD8+ T cells [85]. In line with these observations, in primary murine and human macrophages, HDL promoted a pro-inflammatory response, leading to rapid bacterial clearance by phagocytosis, via passive cholesterol efflux, activating the PKC/NF-kB/STAT1 signaling pathway [207].

Because LDL primarily promotes lipid influx into cells and HDL is involved with the efflux of excess lipid from cells, the findings above suggest that lipid influx into TAMs and lipid efflux from TAMs would polarize TAMs, respectively, into the M2 and an M1 phenotypes. In support of this model, there are reports in the literature that overexpression of SR-B1 dampened the LPS-induced inflammatory response in macrophages while limiting the SR-B1 gene enhanced pro-inflammatory response to LPS [208, 209]. The overall effects of lipoproteins on TAMs, however, are rendered complex by the signal transduction elicited by the interaction of lipoproteins with surface receptors on TAMs and the interaction of TAMs with cancer cells and non-cancer cells in the TME and the hypoxic and acid nature of the TME. Thus, in contrast, few studies have indicated that cholesterol efflux from TAMs can render them pro-tumoral. For example, knocking down the two major cholesterol efflux mediators, ABCA1 and ABCG1, in macrophages increases the M1 pro-inflammatory (anti-tumor) macrophage population in the TME and was found to inhibit tumor progression in murine melanoma and bladder cancer models [210] [211]. In a murine ovarian cancer model, Goossens et al. [212] showed that cancer cells are responsible for cholesterol efflux from TAMs as their hyaluronic acid secretion binds to receptors on the surface of TAMs and contributes to the depletion of cholesterol form lipid rafts. This depletion of cholesterol induced an IL-4, STAT 6 (signal transducer and activator of transcription 6), and phosphoinositide 3-kinase signaling, rewiring TAMs' metabolism and transforming their phenotype toward M2. In this model, TAMs can thus be considered as cholesterol reservoirs that support the proliferation of cancer cells at the expense of their pro-inflammatory signaling which depends on the integrity of lipid rafts. The findings, summarized above, clearly support therapeutic approaches that involve macrophage polarizing (or re-polarizing) agents, delivered via scavenger receptors (and especially SR-B1) that are overexpressed by macrophages.

6.3 Role of Lipoprotein/TME Interactions in Designing Novel Therapeutic Approaches

6.3.1 Barriers Preventing Nanoparticles from Reaching Tumor Targets

To improve the approach of nanoparticles to tumor cells, the fundamentals of drug delivery need to be considered. Nanoparticles navigate the vascular barrier through endothelial cell gaps, partially created by poor lymphatic drainage, a mechanism termed “enhanced permeability and retention” (EPR). The endothelial cells surrounding the tumor cells, subject to rapid growth due to increased VEGF and HIF, may also facilitate nanoparticle extravasation into the cell. However, an analysis of 232 datasets revealed a very poor delivery efficiency, indicating only a median of 0.7% of injected nanoparticles reaching their tumor target [96]. In addition, ligand (trastuzumab)-coated, gold nanoparticles (AuNPs) were found to deliver less than 0.0014% of the injected dose to tumors [97]. Further analysis indicated that tumor-associated macrophages (TAMs) and regions within the solid tumor sequestered >88.2% of extravasated nanoparticles [97], indicating that these types of nanoparticles were 7–38 times more likely to interact with TAMs instead of cancer cells. Cell death was observed in both cancer cells (0.4%) and macrophage (3.6%) populations but more so in macrophage populations. When the nanoparticle dose was increased tenfold, there was an increase in the macrophage cell death (34.3%), but not in the cancer cells [97]. These findings led to speculation that the tumor shrinkage reported in other studies could be due to death of stromal cells and cancer cells indiscriminately rather than suppressing of tumor growth. Furthermore, it is evident that greater selectivity must be attained for nanoparticle delivery mechanisms, resulting in greater efficacy of therapy delivery by ensuring that the payload reaches the specific desired target [97]. A recent review [213] suggests that the types of tumors that are resistant to drug delivery (EPR-insensitive type) could be made accessible to chemotherapy by treatments, including ultrasound and photodynamic therapy.

As nanoparticles are likely to be considered by human tissues as foreign substances, the renal system and macrophages, associated with lymphoid organs or tumors, are likely to be involved in their clearance mechanisms. The macrophages found around cancer cells in the TME are termed tumor-associated macrophages (TAMs), and they play a particularly important role in the processing/disposal of nanoparticles [96]. Thus, to navigate the TME and reach its tumor target, the ideal nanoparticle would have to be small and uncharged and resist detection by macrophages as a foreign substance. Once it has passed these hurdles, it must either resist biological change or permutate in a manner that favors interaction with tumor cells. These criteria characterize lipoprotein-type drug carriers that have already proven to be highly effective during initial proof of concept studies [48] [98, 214].

Regarding the increased uptake (trapping) of nanoparticles by TAMs, their phagocytic properties could promote distribution of these drug-transporting nanoparticles away from cancer cells, their intended target [97]. Nanoparticles may be identified by macrophages as foreign substances and subsequently sequestered and degraded [96]. It has been observed that smaller nanoparticles are cleared at a slower rate than larger nanoparticles. Macrophages preferentially engulf particles with cationic charge, followed by those with anionic surface charge and finally by those with zero net surface charge. This observation has led to coating nanoparticles with neutral polymers as a form of camouflage. The capability of the nanoparticle to evade the macrophage is an important property, as longer half-life will allow an increased accumulation within the tumor. The physiochemical attributes of the nanoparticles are likely to change upon interaction with tissue constituents. In the design of drug-transporting nanoparticles, their properties should provide the appropriate features so that the resulting nanoparticle, subsequent to tissue interactions, is still capable of targeting tumor cells [96].

6.3.2 Lipoprotein TME Interactions

Certain characteristics of the TME enhance cancer cell viability, especially when challenged by anti-cancer treatments. It has been postulated that components of the TME could be significant contributors to drug resistance as well. Consequently, an ideal cancer therapeutic approach would have to include the ability to penetrate the TME and carry out its intended function in order to exert the desired therapeutic effect. Tumor cells create an environment of hypoxia and acidosis that has been correlated with increased treatment resistance and tumor aggressiveness. In hypoxic conditions, there are numerous cellular changes where lipoproteins could play key roles. In glioblastoma cell lines, hypoxia induced the uptake of all three classes of lipoproteins (HDL, LDL, and VLDL) over a wide range of concentrations, as tested by quantitative flow cytometry studies. The effect was observed to be indirect (induced mechanism), as uptake of all lipoprotein classes significantly increased when the cells were preincubated in an acidic environment. There was no additional effect of hypoxia observed on lipoprotein uptake stimulated by acidosis [26]. As lipoproteins enter the intracellular environment, via endocytosis or via other mechanisms, changes in receptor expression, brought about by hypoxia, could be an important determinant in carcinogenesis and metastasis. Lipoproteins and their payloads may be internalized via binding to a variety of cell surface receptors, including VLDLR, LDLR, LRP1, and SR-B1. When laser micro-dissected regions of glioblastoma patient tumors were compared on the basis of hypoxic vs normoxic regions, LDLR was not induced by hypoxia. However, hypoxic induction of VLDLR, SR-B1, and LRP1 mRNAs was observed, with SR-B1 mRNA having the highest induction (fivefold at 24 hours). Hypoxic induction of VLDLR and SR-B1 proteins was quantified using an immunoblotting experiment as well, indicating stress-induced lipid loading into cancer cells in conjunction with upregulated receptor expression [26]. The marked (stress-induced) elevation of SR-B1 receptor expression validates the utility of therapeutic anti-cancer agent delivery via rHDL nanoparticles [3].

Uptake of drug-carrying nanoparticles by non-malignant tissues can be considered to be an off-target, undesired outcome of therapy. However, targeted uptake by tumors is the main goal of therapeutics especially in oncology. A new approach involves coating nanoparticles with macrophage membranes, to selectively target tumors in breast cancer and lung cancer [215]. Macrophage cell membrane has been used to decorate gold nanoparticles, allowing the nanoparticles to target the tumor and remain in circulation longer, as tested in vivo. Stability of the macrophage coating is of concern, as shedding of the exterior macrophage membrane coat may result in additional unintended off-target effects [216].

6.4 Conclusion

The amount of information currently available in the literature regarding TME/lipoprotein interactions is very limited. We attempted to review all relevant information that might lead to improved diagnostics or therapeutics of solid tumors, at times a speculative venture, in addition to the published reports dealing with the potential role(s) that lipoprotein may play in regulating events in the TME.

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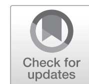

7 The Role of BEHAB/Brevican in the Tumor Microenvironment: Mediating Glioma Cell Invasion and Motility

Kristin A. Giamanco and Russell T. Matthews

Abstract

Malignant gliomas are the most common tumors in the central nervous system (CNS) and, unfortunately, are also the most deadly. The lethal nature of malignant gliomas is due in large part to their unique and distinctive ability to invade the surrounding neural tissue. The invasive and dispersive nature of these tumors makes them particularly challenging to treat, and currently there are no effective therapies for malignant gliomas. The brain tumor microenvironment plays a particularly important role in mediating the invasiveness of gliomas, and, therefore, understanding its function is key to developing novel therapies to treat these deadly tumors. A defining aspect of the tumor microenvironment of gliomas is the unique composition of the extracellular matrix that enables tumors to overcome the typically inhibitory environment found in the CNS. One conspicuous component of the glioma tumor microenvironment is the neural-specific ECM molecule, brain-enriched hyaluronan binding (BEHAB)/brevican (B/b). B/b is highly overexpressed in gliomas, and its expression in these tumors contributes importantly to the tumor invasiveness and aggressiveness. However, B/b is a complicated protein with multiple splice variants, cleavage products, and glycoforms that contribute to its complex functions in these tumors and provide unique targets for tumor therapy. Here we review the role of B/b in glioma tumor microenvironment and explore targeting of this protein for glioma therapy.

Keywords

Proteoglycan · Glioma dispersion · Glycosylation · ADAMTS4 · MMP · Lectican · Chondroitin sulfate · Fibronectin · Glioma-initiating cells · EGF receptor · Hyaluronan · ECM · TME · BEHAB · Glycoform

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7.1 Introduction

The tumor microenvironment is comprised of the cells that directly make up the tumor, neighboring normal/non-transformed cells, the extracellular matrix (ECM), and secreted molecules

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found within this space [5, 52, 140]. Importantly, interactions between these constituents define the molecular properties of the specific tumor [52]. High-grade gliomas are the most frequently detected and virulent form of intracranial tumors, but they are also commonly impervious to currently available treatments, including surgery as well as chemotherapy and radiation [125]. One of the primary reasons as to why gliomas are insusceptible to these therapies is due to the fact that gliomas are able to infiltrate surrounding tissues and, thus, are considered to be highly invasive [109, 126].

Many of the components within the tumor microenvironment support the dispersion and heightened motility of glioma cells. Specifically, gliomas exhibit aberrant expression patterns of cell adhesion molecules, ECM molecules, proteolytic enzymes that remodel the ECM, and growth factors [5, 140]. One such ECM molecule that is overexpressed in gliomas is the chondroitin sulfate proteoglycan (CSPG): brain-enriched hyaluronan-binding protein (BEHAB)/brevican (B/b) [42, 67]. This enhanced expression of B/b leads to an increase in the aggressiveness of the resulting tumors [35, 62, 83, 98, 134, 154]. It is important to note that that there are a number of B/b isoforms that are upregulated in glioma samples [133].

Mechanistically, B/b is cleaved by a disintegrin and metalloproteainase with thrombospondin motifs (ADAMTS)-4 [86], and this causes the abnormal adhesion of glioma cells to fibronectin, and the overall motility of the glioma cells is enhanced [62], thereby leading to tumor invasion. Moreover, as a result of an increase in the presence of B/b, fibronectin secretion is increased, as is the expression of a number of cell adhesion molecules [62]. Taken together, these molecular and structural changes to the tumor microenvironment favor glioma cell invasiveness and movement, which contributes to the resistance of gliomas to current therapeutics [109].

In this chapter we first focus on the function of B/b in normal/non-transformed cells and then delve into the molecular composition of the tumor microenvironment. Next, we dissect the specific role that B/b plays in promoting glioma cell invasion and motility. Additionally, the mechanisms underlying these processes will be discussed and will shed further light onto why the current treatments are not more effective at targeting gliomas and why targeting B/b could be a new therapeutic strategy.

7.2 Brevican: Structure and Function

7.2.1 Structure

B/b is a member of the lectican family of CSPGs along with versican, neurocan, and aggrecan [7, 113, 147]. In terms of structure, members of the lectican family are quite homologous. More specifically, these CSPGs contain a N-terminus, which is known as the hyaluronan (HA)-binding domain and link protein-like region, that mediates interactions between the lectican family members and HA (see Fig. 7.1, [104]). The binding of these CSPGs to HA is a key step in overall organization of the ECM. Within the C-terminus, there is an epidermal growth factor (EGF)-like domain that is characteristic of proteins found within the ECM, a lectin-like domain, and a complement regulatory protein-like domain [120]. It is through this lectin domain that the lecticans can bind tenascin-R, a glycoprotein present within the ECM. Furthermore, this region of CSPGs can also bind to glycolipids found on the cell surface that have been sulfated, which promotes cell adhesion [89].

CSPGs consist of a core protein that is decorated with chondroitin sulfate (CS) sugar chains, which bind to the protein in the CS attachment region (Fig. 7.1). The amount of sugar units that can be added to the protein varies widely between members of the lectican family with B/b having 1–3 CS chains that adorn the protein core [7]. The core protein has been shown to hinder neurite outgrowth in cultured neuroblastoma cells [64], and the CS region of the CSPG has also been reported to inhibit overall growth and regeneration in the central nervous system (CNS) [21, 70]. A substantial body of work utilizing the bacterial enzyme, chondroitinase ABC (chABC), to

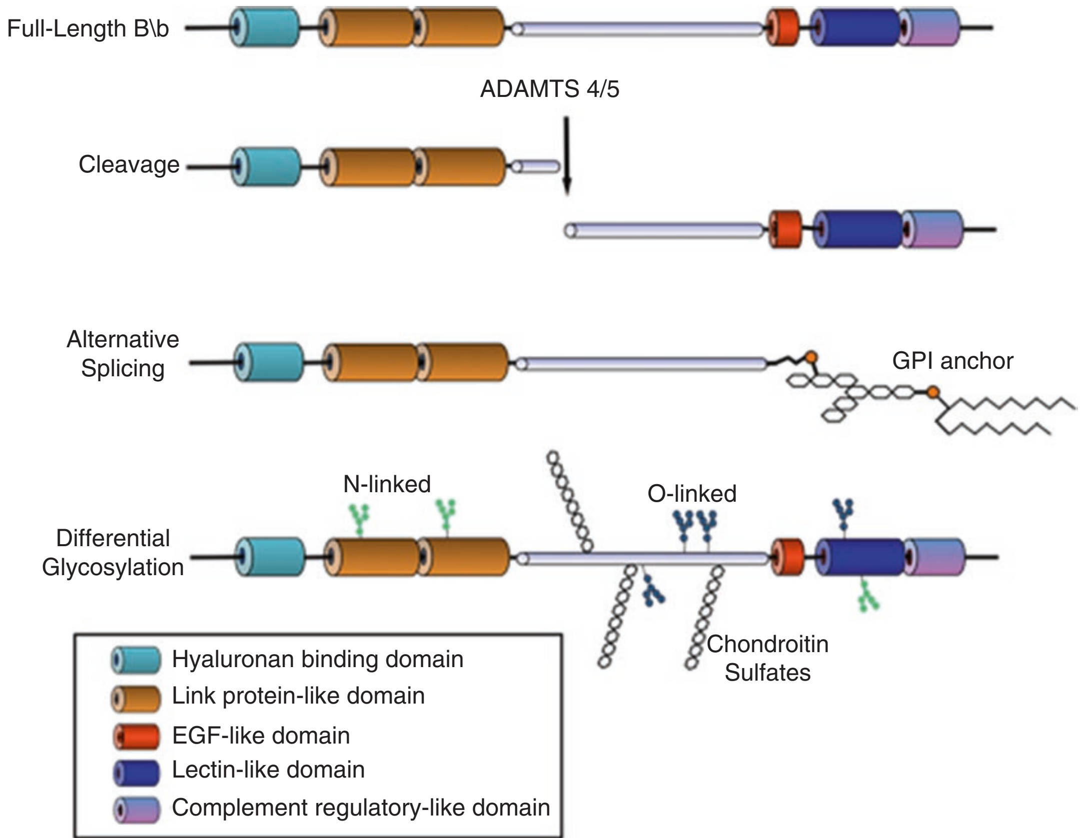

Fig. 7.1 BEHAB/brevican structure diversity, glycosylation, and cleavage. B/b is made as a both a secreted and GPI-linked isoform. It is routinely cleaved at a defined ADAMTS4/5 cleavage site leading to N-terminal and C-terminal fragments. In addition, work has shown that there is a lot of microheterogeneity in the glycosylation of this protein. In gliomas all forms of B/b are upregulated with the N-terminal cleavage fragment perhaps being the most critical functionally. In addition there are glioma-specific glycoforms that are generated that may provide ideal targets for glioma therapy

digest CS chains supports these findings. In these studies, application of chABC resulted in recovery after spinal cord injury [14, 75, 136]. Additionally, administration of chABC has been reported to enhance axonal regeneration within the CNS in undamaged animals [31, 90]. Pizzorusso and colleagues used this enzyme to reopen the critical period and, thus, restore plasticity within the visual system of adult rats [105]. Taken together, both the core protein and the CS region of CSPGs inhibit growth and regeneration within the CNS and thus play a key role in restricting overall brain plasticity.

Specifically, B/b can exist in a number of different ways: a glycosylphosphatidylinositol form that is anchored to the plasma membrane [119, 120] and a form that is secreted right into the ECM [119], and additionally, B/b can be present as a glycosylated proteoglycan or as a core protein that is not glycosylated (Fig. 7.1, [146]). Interestingly, the form that is anchored to the plasma membrane was detected primarily in white matter tracts where axons are located as well glial cells that were classified as diffusely distributed throughout the brain. The form that is secreted into the ECM was highly expressed in the gray matter within the cerebral cortex, hippocampus, cerebellum, and particular thalamic nuclei [119]. Through Western blot analysis, in the adult rat brain, the full-length B/b protein

runs at 145 kDa, but cleavage products have been described at 90 kDa and 50 kDa [145, 154].

Temporally, B/b expression is first detected on embryonic day 15 (E15). In all assayed regions of the CNS, the onset of B/b expression occurred after neurogenesis and instead was consistent with the generation of glial cells [66]. It has also been demonstrated that B/b expression is upregulated in response to injuries within the brain [41]. Following a stab wound to the adult rat brain, B/b was detected in regions of active gliosis [65], and, similarly, B/b expression within astrocytes was increased in response to lesions introduced into the entorhinal cortex in rats [127]. In that same vein, B/b mRNA was dramatically increased within the glial scar following cryo-injury in mice [64].

7.2.2 Function

B/b is one of the molecular constituents of the perineuronal net (PNN) that is found within the CNS. PNNs surround the cell body and proximal neurites of particular populations of neurons within the CNS. Typically, these cells are GABAergic interneurons, but they also can be found around excitatory cells. This structure serves to restrict plasticity by closing the critical period [9, 17, 23, 24, 48, 53, 58, 59, 124, 153]. Other work postulates that PNNs provide a buffering mechanism to preserve the balance of cationic charges within the extracellular milieu [17, 18, 54, 55]. Using B/b deficient mice, Bekku and colleagues revealed that B/b regulates the assembly of the proteoglycan, phosphacan, and tenascin-R at Nodes of Ranvier within the CNS [8], which is likely critical in action potential propagation. Proper B/b expression is also needed to maintain normal speeds of synaptic transmission at the calyx of Held in the medial nucleus of the trapezoid body within the brainstem, where PNNs are found in abundance [13]. Studies performed using B/b knockout mice highlight a potential role for B/b in modulating long-term potentiation in the CA1 region of the hippocampus. Of particular interest, the mice lacking B/b displayed less prominent PNNs, meaning that they were less condensed and focused at the cellular surface and instead exhibited a more diffuse expression pattern [15].

7.3 Gliomas

7.3.1 Invasion and the Tumor Microenvironment

The tumor microenvironment describes the environment around a particular tumor and consists of both cellular and non-cellular components [5, 52, 140]. It is important to note that the tumor cells and the other constituents of the microenvironment interact with one another, and this can influence the growth and spread of the tumor [52]. In gliomas their most conspicuous ability is their invasive properties within the central nervous system, which are typically very inhibitory to cellular movement.

The high mortality rate of patients with high-grade gliomas is explained by the fact that gliomas uniquely invade the central nervous system [109, 126]. The neural ECM is usually thought of as an inhibitory environment, one that is not conducive to large-scale reorganization or remodeling; this has been attributed to the high presence of CSPGs [107, 113]. Gliomas are able to circumvent this inhibitory barrier. One of the main ways gliomas are able to do this is through the secretion of molecules that facilitate cell adhesion and movement, which include fibronectin and collagen [12, 22, 30, 44, 45, 99, 106]. Other ECM molecules have been demonstrated to regulate the phenotypic characteristics of gliomas including laminin, vitronectin, and tenascin-C. Using in vitro assays, it has been reported that glioma cells produce and secrete laminin [87, 103]. Expression of the glycoprotein, vitronectin, is correlated with the glioma grade, and its expression has been linked to increased cell survival of glioma cells [131]. Similarly, tenascin-C expression is also linked to glioma grade, and its expression is thought to be involved in mediating cell adhesion, migration, and cell dispersion [57,

152]. Gliomas have also been shown to contain high levels of other ECM components like osteopontin, secreted protein acidic and rich in cysteine (SPARC), and thrombospondin [10]. Expressions of B/b, neurocan, and versican are also increased in glioma samples [100, 132, 134].

Enhanced expression of MMPs is characteristic of many tumor types, including gliomas. These enzymes degrade parts of the ECM, which then allows for the glioma cells to move through the ECM and infiltrate surrounding tissues. The MMPs that are upregulated in glioma cells include MMP-2, 3, 7, 9, 12, 13, 14, 16, 19, and 26 [33, 46, 63, 71, 73, 76, 85, 92, 106, 111, 115, 117, 118, 137, 138, 141, 148–150]. Other enzymes that are also responsible for the invasive properties of glioma cells are cathepsin B and urokinase-type plasminogen activator [11, 46, 71, 106, 115, 118, 141, 149] and heparanases and sulfatases [82]. In human gliomas, the overexpression of the forkhead box m1b (Foxm1b) factor leads to the enhanced invasion of glioma cells through an increase in transcription of the MMP-2 gene [32, 80].

Growth factors like epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) have been reported to mediate glioma cell invasion [28]. Glioma cells commonly display mutations or amplifications in the EGF receptor (EGFR) gene, and there is an increased presence of this receptor on the surface of the tumorigenic cells [81, 96]. Interestingly, the activation of EGFR and extracellular signal-regulated kinase (ERK) is thought to result in an increase in the expression of fibronectin [38, 130, 155], which likely underlies the increase in migration exhibited by glioma cells. Hepatocyte growth factor (HGF) is commonly overexpressed in glioma cells, and, as a result, cell migration pathways are activated, which leads to the enhanced movement of these cells [47]. Similarly, insulin-like growth factor (IGF) is also overexpressed in these tumorigenic cells, and, in specific, an increase in expression of IGFBP2 leads to an upregulation of genes that are involved in cancer cell invasion, including MMP-2 [139]. High levels of the angiogenic factor, angiopoietin-2 (Ang2), are detected in more invasive areas of gliomas, and this enhanced expression induces upregulation of MMP-2 both in vivo and in culture assays [50, 61, 69, 71]. The cell surface chemokine receptor, CXCR4, is also highly expressed in invasive glioma cells [36], and when the receptor interacts with a specific ligand, the Akt and ERK1/2 signaling pathways are activated, which affords glioma cells an increase in survival and cell division. This results in a more invasive phenotype [112, 142].

The canonical hyaluronan receptor, CD44, activates Rac1, which leads to a dramatic restructuring of the actin cytoskeleton within glioma cells. This receptor can be cleaved by ADAMTS10, and the product increases the invasive properties of glioma cells [3, 10, 91]. Rac not only facilitates the rearrangement of the actin cytoskeleton but is also known to increase cell motility [16, 110]. To demonstrate this, investigators inhibited Rac expression and found that glioma cell invasion was decreased [26, 29]. Rac does not work independently to mediate such important events; it has been reported that Rac works with the polypeptide P311 [84, 88]. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is detected at high levels in glioma cells and has been hypothesized to afford these glioma cells an enhanced cell survival rate [114].

Glioma cells also exhibit changes in expression of cell adhesion molecules. For example, glioma cells express focal adhesion kinase (FAK) at higher levels than non-tumor cells [51, 56, 135], which has been linked to increases in cell proliferation [79, 151]. On the other hand, some cell adhesion molecules may exhibit decreased levels of expression in glioma cells. Expression of neural cell adhesion molecule (NCAM), for example, is reduced in glioma cells, which allows them to separate from neighboring cells and disperse into surrounding tissues [101, 116]. Cell surface integrin receptors that help join cells to one another are upregulated in glioma cells; specifically, this includes integrin α3β1, αvβ1, αvβ3, and αvβ5 [74]. Other cell adhesion molecules that display abnormal expression patterns in gli-

oma cells include adhesion molecule on glia/β2 subunit of Na, K-ATPase (AMOG/β2), ephrin receptor tyrosine kinases (EphB2–B3), fibroblast growth factor inducible 14 receptor (Fn14), and protein tyrosine phosphatases zeta/beta [37, 94, 95, 121, 129]. Cadherin molecules also work by joining adjacent cells to one another to form structures like adherens junctions. Any changes to the structure and stability of these junctions result in an increase in the movement and invasiveness of glioma cells [4]. Glioma cells that express high levels of E-cadherin are phenotypically highly invasive [77], while cells that contain high levels of N-cadherin demonstrate the opposite, in that they are less invasive [19, 93].

7.3.2 Glioma-Initiating Cells (GICs)

Gliomas are highly resistant to current therapeutic interventions, and, as a result, patient mortality rates are still quite high [126]. The invasive properties of gliomas are key in their therapeutic resistance; however, also key is the existence of glioma-initiating cells (GICs) within these tumors. In terms of the cellular composition, GICs that are present within the tumor are molecularly distinct from other cells found within the glioma. More specifically, the GICs are capable of self-renewing and exhibit multipotency, which means that they can differentiate into any subpopulation of cells found within the CNS as well as within the tumor itself. After orthotopic transplantation, these stem cells possess the ability to form a tumor that physically matches the parental tumor [25, 39, 123].

The GICs express many of the same proteins as those that are detected within normal stem cell niches [34] including laminin [122], tenascin-C [2, 40], members of the lectican family of CSPGs [68], and phosphacan [1, 2] as well as members of the integrin family [122]. Furthermore the GICs tend to be localized near vasculature within the tumors [43, 122]. It is important to note that the vasculature may develop due to the tumor presence itself, or the GICs might possess the ability to differentiate into endothelial cells, thus forming new blood vessels [108].

7.3.3 A Key Role for B/b in the Glioma Microenvironment

As noted above the interactions with tumors are complex within the tumor microenvironment; however, B/b presents as a uniquely intriguing target within this complex environment, and its specific roles are detailed below.

7.4 The Role of B/b in Gliomas

7.4.1 B/b Expression in Gliomas

Enhanced levels of B/b expression have been detected in human glioma samples, including oligodendrogliomas, all examined grades of astrocytomas, and gliosarcomas, relative to normal brain tissue and tissues derived from non-glioma tumors [42, 67]. More specifically, this increase in B/b expression was detected within the ECM as well as the cytoplasm of glioma cells. Importantly, within higher grades of astrocytomas (grades III and IV), B/b staining was more dispersed, indicative of an increase in infiltration, compared to lower grades [83]. Glioma cell lines (e.g., 9L, CNS-1, and C6) that are propagated under normal culture conditions do not express B/b, but if they are grown as intracranial grafts, then they express B/b. This phenomenon was not noted in cells taken from noninvasive tumors [67].

In rodent and human glioma samples, B/b is cleaved, and the resulting products are a N-terminal fragment that includes the hyaluronan-binding portion of the protein core (~50–60 kDa) and a C-terminal fragment (~90–100 kDa). Full-length B/b runs at ~160 kDa [133, 154].

Gary and colleagues set forth the hypothesis that B/b modulates the invasiveness of gliomas [41]. To examine the properties and behaviors of gliomas further, investigators introduced B/b into CNS-1 cells in vitro through transfection. These cells were transfected either with a green fluorescent protein (GFP) control, full-length B/b, the C-terminus cleavage fragment of B/b, or the N-terminus cleavage fragment of B/b. These cells

were then injected into rats to assess the resulting tumors. The rats that were injected with the CNS-1 cells transfected with the various forms of B/b, exhibited a lower survival rate than those rats that received the control cells. Furthermore, the B/b-derived tumors were more invasive and were highly vascular compared to the control tumors. This led to the conclusion that B/b enhances the aggressive properties of gliomas [98].

While it was established that full-length B/b is overexpressed in human glioma samples [42, 67], it was then identified that two novel isoforms of B/b were present in tumor tissues [133]. The two new isoforms were denoted as $B/b_{\Delta g}$ with a molecular mass of 150 kDa that was found within the membrane fraction and B/b_{sia}, which had a molecular weight higher than 150 kDa and was located in both the membrane and soluble fractions. It is important to note that the benign tumors that were assayed did not express these B/b isoforms, thereby indicating that perhaps these identified forms of B/b could be used as indicators of tumor grade. Furthermore, these isoforms were specifically identified in gliomas and were not present in tissues derived from patients with epilepsy or Alzheimer's disease. Neither isoform of B/b was found within individuals over 1 year of age. Only the $B/b_{\Delta g}$ form was faintly detected in samples harvested from embryos at 16 weeks of gestation to infants aged 19 days [133].

Through biochemical analyses, it was determined that these new isoforms of B/b were not cleavage fragments, and the peptide sequences of these forms were identical to the full-length protein, thereby indicating that these forms were derived from the same mRNA transcript. Attention was then focused on determining how these isoforms were molecularly distinct from the full-length protein. Deglycosylating enzymes were used to remove N-linked and O-linked sugars from the protein core as well as CS chains (through the use of chondroitinase), and this revealed that the $B/b_{\Delta g}$ form was underglycosylated. Further work determined that this particular isoform associates with the cell membrane in a manner distinct from other B/b forms. Importantly, this does not require calcium, interaction with the CS chains, nor the N-terminal domain of B/b, affirming that the molecular association with the membrane is unique for this specific isoform. The B/b_{sia} form is an over-sialylated version of the protein and is generated when there is an increase in the amount of sialic acid added to O-linked carbohydrates [133].

Having established that B/b is expressed at high levels in gliomas, the next step was to ascertain which specific cellular population contained the highest amounts of B/b. Human glioblastoma tumor sections were analyzed, and B/b was found around cells that expressed Olig2 and CD133 markers, both of which are indicative of highly tumorigenic cells [20, 49]. Interestingly, these markers are also detected within GICs. To examine B/b expression in these cells, researchers utilized two GIC lines: 0627 and 0913. They determined that both B/b protein and mRNA were present in these cell lines, although the 80–90 kDa C-terminal cleavage fragment was only expressed in the 0627 cells. Interestingly, B/b knockdown did not alter any of the assayed physical properties of the glioma-initiating cells including proliferation rate, viability, adhesive properties, migration, and invasion. Based on these results, it does not appear that B/b is needed for the GICs to behave normally nor for the maintenance of their characteristic physical properties, so likely B/b works in this cell population during the later stages of glioma pathogenesis [35].

7.4.2 Cleavage of B/b in Gliomas Leads to Increased Invasiveness

To address the ability of B/b to promote invasiveness, cultured 9L cells were transfected with either the full-length protein or the N-terminal fragment described above. It is important to note that the 9L cell line is characterized as a noninvasive cell line. 9L cells that expressed either the full-length form of B/b or the N-terminal fragment displayed a higher degree of motility and invasion as compared to cells that were trans-

fected with GFP. Of particular importance, when these cells were injected into rats, only the tumors that expressed the N-terminal cleavage fragment were able to invade surrounding brain tissue. This was not noted in tumors that expressed the full-length form of B/b, thereby suggesting that the cleavage of B/b is a key event that mediates glioma cell invasiveness in rat models [154].

This finding prompted the investigation into which molecule cleaves the lectican family member. This was addressed through the generation of an antibody against the putative cleavage site at Glu^{395}-Ser^{396} within B/b [86]. The cleavage site is homologous to the well-characterized site in another CSPG, aggrecan [156]. Cleavage of aggrecan at that particular site is regulated at least partially by ADAMTS4 [128].

The resulting antibody exclusively recognizes the N-terminal fragment of B/b and is referred to as B50. Through use of the invasive CNS-1 cell line, cleavage activity was detected in culture, and most of the resulting product was detected in the media and, thus, was soluble. Investigators then aimed to determine the proper conditions for B/b cleavage by altering calcium, zinc, and sodium chloride levels in addition to pH and temperature. Administration of calcium chelators, and metalloproteinase inhibitors to the cultures, diminished the cleavage of B/b. From this work, Matthews and colleagues examined the potential role of ADAMTS4 in mediating the cleavage of B/b. They concluded that ADAMTS4 not only was expressed in CNS-1 cells but was also capable of cleaving B/b. This work pinpoints a critical role for ADAMTS4 to regulate B/b cleavage and, by extension, the invasive behavior displayed by glioma cells [86].

To directly assess if this cleavage event is necessary for the pro-invasive properties seen in glioma cells, a mutant construct in which B/b was not cleaved was introduced into CNS-1 cells. Tumor spheroids were created and then applied to organotypic slice cultures, and migration of the cells was examined. The spheroids containing the wild-type form of B/b migrated across the slice cultures more than those that expressed the mutant form of B/b that could not be cleaved. The CNS-1 cells that were transfected with wild-type B/b were implanted into rats intracranially, and the resulting tumors were more invasive, dispersed, and larger compared to those tumors that formed when the CNS-1 cells transfected with the mutant form of B/b were injected. Rats that had tumors that were more invasive exhibited a decreased survival rate compared to their counterparts [134].

7.4.3 Molecular Mechanisms: How B/b Cleavage Promotes Invasiveness

Having determined that the cleavage of B/b promotes glioma cell invasion, the mechanisms underlying this were next explored. B/b was introduced into glioma cells (U87MG, U373MG, and CNS-1 cells) through transduction in culture. Expression of B/b enhanced glioma cell adhesion to specific substrates: fibronectin, collagen, and hyaluronic acid, but this was not noted when laminin and poly-L-lysine were used. Moreover, investigators probed glioma cell motility and reported that glioma cells expressing B/b were more mobile in response to hyaluronic acid and fibronectin substrates, in comparison with control cells that did not express B/b. B/b cleavage was required for the adhesion between B/b and fibronectin and hyaluronic acid. To provide further support, the glioma cells were added to organotypic slice cultures to measure the amount of cell dispersion. Glioma cells that expressed either the full-length form of B/b or the N-terminal cleavage fragment of B/b exhibited a significant increase in cell movement compared to those cells that expressed the form of B/b that was resistant to cleavage [62].

The expression of a number of cell adhesion molecules is altered in glioma samples [4, 19, 37, 51, 56, 74, 77, 79, 93, 94, 95, 101, 121, 129, 135, 151, 157], and Hu and colleagues then focused on identifying which cell adhesion molecules might be involved in modulating glioma cell invasion. Glioma cells that expressed B/b and were plated on fibronectin displayed an increase in protein expression of β-3 integrin, a phosphorylated form of the β-3 integrin, and NCAM, in comparison

with control cells that did not express B/b [62]. These results are in accordance with reports that β-3 integrin expression induces cell dispersion and spreading [143, 144]. It is known that both B/b and fibronectin are upregulated in gliomas [99, 133] compared to normal brain tissue [97, 102] and tumors that spread to the brain, but did not originate in the brain [67]. Specifically, fibronectin was found at the cell surface on glioma cells that possessed either the full-length form of B/b or the N-terminal cleavage fragment of B/b. The expressed fibronectin was organized in microfibrillar structures, which is thought to facilitate rearrangement of the ECM and promote movement of tumor cells [60, 72].

When U87MG glioma cells were incubated in conditioned media that contained either secreted full-length B/b or the N-terminal cleavage fragment of B/b, there was an increase in the amount of phosphorylated EGFR and phosphorylated ERK1/2 compared to control cells. If the glioma cells were treated with an EGFR inhibitor, then phosphorylation was inhibited, and, correspondingly, fibronectin mRNA levels decreased. Importantly, as a result of the treatment with this inhibitor, the B/b-expressing glioma cells did not adhere as well to fibronectin relative to cells that were treated with a control empty vector [62]. This work is consistent with reports that the expression of EGFR is increased in glioma cells [81, 96]. Additionally, the results presented by Hu et al. [62] corroborate previous demonstrations that the activation of EGFR and ERK induces an increase in fibronectin expression [38, 130, 155]. Precisely how fibronectin and B/b might associate with one another was directly addressed through co-immunoprecipitation and dot blot assays, in which it was shown that fibronectin binds to the N-terminal cleavage fragment of B/b, but not the full-length form of the protein. This clearly shows that the cleavage of B/b is an important event that is required for binding to fibronectin, which results in the enhancement of glioma cell movement [62].

The work presented by Hu and colleagues was supported by another set of experiments where U251 and U87 glioma cells were induced to express B/b, which resulted in an increase in the adhesion of glioma cells to fibronectin and an overall increase in motility of the glioma cells [83].

7.4.4 Impact of B/b Knockdown on Glioma Cells

To further pinpoint the critical role that fibronectin plays in mediating glioma cell motility, siRNA constructs were made to knockdown fibronectin expression. As a result, glioma cells adhered less to hyaluronan and fibronectin and additionally were less motile when plated on these substrates. Phenotypically, these glioma cells now presented the same as the control cells in terms of adhesion and motility [62].

To more thoroughly analyze how B/b is involved in regulating glioma cell behavior, U251 cells were first transduced to express B/b, and then the protein was knocked down using shDNA. Due to the knockdown of B/b, these glioma cells displayed a decrease in the rate of division and reductions in the following properties: invasiveness, migration, and dispersion or spreading distance, in direct comparison to the shDNA and mock controls. To examine resulting tumor growth in vivo, the transduced cells were introduced into nude mice. The mice that received the B/b knockdown cells developed tumors that were less infiltrative and less dispersed relative to the mice that received the control cells. This work further defined the role of B/b role in regulating glioma cell migration and invasion [83].

Dwyer and colleagues then aimed to elucidate what occurs when B/b is knocked down in intracranial gliomas. To this end, investigators transfected CNS-1 cells with B/b expression constructs at the same time as B/b knockdown constructs. After determining the knockdown efficiency, the generated CNS-1 cells were injected in the thalamus of rats. The tumors that formed after CNS-1 cells exposed to the shRNA construct specific to B/b displayed a reduction in overall volume and were less invasive compared to the tumors that developed when a control shRNA construct was introduced into the CNS-1 cells. In light of this, the survival rates of the rats injected with the B/b

knockdown cells were higher than those rats that received the control cells [35]. This body of evidence suggests that B/b expression in gliomas results in increased motility and invasion [35, 83].

As stated above, B/b expression was detected in GICs [20, 35, 49], but the question as to how B/b functions in this cell population was next addressed. shRNA constructs were introduced into GICs and then were injected into the striatum of nude mice to examine the properties of the tumors that were generated as a result. The GICs that expressed the knockdown constructs to reduce B/b expression formed a tumor that was smaller in volume and was less invasive relative to the cells that contained control constructs [35]. Therefore, it does appear that B/b mechanistically functions in the same capacity in the glioma-initiating cells as in glioma cells to promote invasion, spread, and migration.

7.5 Future Directions

B/b is a key molecule present within the tumor microenvironment of gliomas that works to promote cell invasion and movement [35, 41, 62, 83, 86, 98, 133, 134, 154]. Due to the fact that glioma cells possess the ability to infiltrate the normally inhibitory ECM, patient prognosis and response to current treatment options are quite poor [126]. In addition, work suggests that B/b contributes to tumor vascularization, but the mechanism by which it does this is completely unknown [98]. Future work investigating the interaction between B/b and other cells in the tumor microenvironment such as pericytes and vascular endothelial cells is clearly necessary. In addition, future studies need to be aimed at creating treatments that specifically target the GIC population. These cells are capable of self-renewal and also are able to form all of the cells within a glioma [39, 123]. Importantly, these cells create and maintain an environment that fuels tumor development, which not only is likely responsible for driving the initial establishment of the tumor but also explains why relapses might occur [6, 27, 78]. Therefore, treatments tailored to targeting the GICs might provide promising new avenues that could lead to better patient survival rates. B/b is an intriguing target in this regard as it seems to be an important component of the stem cell niche.

A complicating factor in treating individuals with gliomas is the fact that there is a great degree of molecular heterogeneity in these types of tumors. More specifically, cell adhesion molecules, ECM constituents, enzymes, and growth factors are just some examples of molecules that may underlie glioma pathogenesis. Importantly, these molecules work together to create an intricate and complex tumor microenvironment. In light of this, the best way to devise treatments is to precisely pinpoint how these molecules work together to contribute to the development and maintenance of gliomas in addition to defining the specific role of each of these molecules. This will give us a more complete picture as to how these tumors develop, thereby, providing us with the information necessary to generate more effective treatments.

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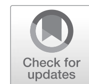

8 Thrombospondin in Tumor Microenvironment

Divya Ramchandani and Vivek Mittal

Abstract

Thrombospondins (TSPs) are multifaceted proteins that contribute to physiologic as well as pathologic conditions. Due to their multiple receptor-binding domains, TSPs display both oncogenic and tumor-suppressive qualities and are thus essential components of the extracellular matrix. Known for their antiangiogenic capacity, TSPs are an important component of the tumor microenvironment. The N- and C-terminal domains of TSP are, respectively, involved in cell adhesion and spreading, an important feature of wound healing as well as cancer cell migration. Previously known for the activation of TGF-β to promote tumor growth and inflammation, TSP-1 has recently been found to be transcriptionally induced by TGF-β, implying the presence of a possible feedback loop. TSP-1 is an endogenous inhibitor of T cells and also mediates its immunosuppressive effects via induction of Tregs. Given the diverse roles of TSPs in the tumor microenvironment, many therapeutic strategies have utilized TSP-mimetic peptides or antibody blockade as anti-metastatic approaches. This chapter discusses the diverse structural domains, functional implications, and anti-metastatic therapies in the context of the role of TSP in the tumor microenvironment.

Keywords

Thrombospondin · Cancer · Microenvironment · CD36 · CD47 · Angiogenesis · TGF-β · Adhesion · Spreading · Invasion · Migration · Dormancy · Pre-metastatic niche · Metastasis · Immune system

8.1 Introduction

Thrombospondins (TSPs) are extracellular, calcium-binding, oligomeric, adhesive glycoproteins that mediate cell-cell and cell-matrix interactions [1]. All TSPs (TSPs 1–5) are expressed at varying levels in many cell types, including endothelial cells, fibroblasts, smooth muscle cells, adipocytes, and macrophages, and exist as a component of the extracellular matrix [2, 3]. TSP-1 promoter has binding sites for transcription factors (OCT-1, IRF-1, AP2, Egr-1, STAT1, PPAR, ATF-1, etc.), indicative of a complex reg-

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ulatory mechanism [2]. TSP upregulation has been observed in physiological processes, including wound healing and repair [4–6], and has emerged as a major player in carcinogenesis [7, 8]. TSPs exhibit context-dependent functional roles; for example, TSP-1 has an inhibitory effect on melanoma growth [9], whereas TSP3 is associated with worse prognosis in osteosarcoma [10]. TSP4 has been associated with tumor suppression in colorectal cancer [11] and increased invasion in breast cancer [12]. TSP2 is associated with increased metastasis in prostate cancer [13] and lung cancer [14] yet exhibits an inhibitory role in angiogenesis and tumor growth in squamous cell carcinoma [15]. Here, we summarize the structural aspects of TSPs, with specific emphasis on the role of TSP-1 in the tumor microenvironment. For general descriptions of TSP in cancer progression, readers are referred to several excellent reviews [16–18].

Thrombospondin, specifically TSP-1, has a multifaceted role in the tumor microenvironment which regulates cancer progression. In addition to its effects on tumor cells, TSP-1 affects tumor stromal cells including endothelial cells, fibroblasts, macrophages, dendritic cells, and T cells. TSP-1 mediates these effects via the interaction with its receptors, CD36 and CD47, or through direct regulation of TGF-β and impacts key signaling pathways. Various functional roles of TSP-1 in the tumor microenvironment are shown in Table 8.1.

8.2 Thrombospondin Structure

TSP-1 is a multifunctional protein, and its diverse biological activities have been mapped to specific domains that interact with different cell surface receptors. TSPs have a conserved carboxyl-terminal “signature domain” which consists of an EGF-like domain, 13 calcium-binding type 3 repeats, and a region homologous to L-type lectin domain. The amino terminus is variable by virtue of possessing a laminin G-like amino-terminal domain and a helical coiled-coil domain that is responsible for the oligomerization. The oligomerization of TSP subunits is mediated by disulfide bonds between adjacent cysteine residues [46]. Based on their oligomeric assembly, TSPs are classified into *subgroup A*. Trimers, TSP-1 and TSP-2, and *subgroup B*. Pentamers, TSP-3, TSP-4, and TSP-5 (or COMP). An array of multiple repeats including *TSP type 1* repeats (TSR1), *TSP type 2* repeats (TSR2, EGF-type repeats), and *TSP type 3* repeats (TSR3, seven continuous calcium-binding repeats) exist between the globular carboxyl and amino-terminal domains in TSPs (Fig. 8.1) [47]. TSPs in subgroup B lack type 1 TSRs present in TSP-1 and TSP-2. Since TSRs are linked to antiangiogenic activities [48–50], TSPs in subgroup B are believed to lack antiangiogenic properties.

8.3 Thrombospondin Receptors

The numerous domains in TSP (Fig. 8.1) mediate a wide array of functions by virtue of binding to different classes of cell surface receptors described below.

8.3.1 Low-Density Lipoprotein Receptor-Related Proteins, Sulfatides, and Proteoglycans

The amino-terminal domain (residue 1-214) of TSP binds to low-density lipoprotein receptor-related protein (LRP) to mediate its internalization and degradation, and this receptor-mediated endocytosis requires heparan sulfate proteoglycans [51, 52]. The N-terminal also binds to a group of anionic molecules, the sulfatides [53]. The interactions involving sulfated glycoproteins and heparin-binding domain in TSP are critical in melanoma cell spreading [54]. Melanoma cell lines that produce glucuronosyl 3-sulfate-containing glycolipids and glycoproteins are selectively capable of spreading via binding to TSP, whereas C32 melanoma cells which do not produce these sulfated glucuronosyl oligosaccharides lack spreading [55].

Table 8.1 Functional roles of TSP-1 in tumor microenvironment

TSP-1 function	Cell-type affected	Effect	Mechanism	Reference
Adhesion	Cancer cells	Increased adhesion Decreased adhesion	Integrin, sulfated glycoconjugates or CD36-mediated TSP-1 adhesion Upregulation of urokinase plasminogen activator receptor	[19–24]
Invasion and migration	Cancer cells Endothelial cells	Increased motogenic effects	Increased MMPs expression via integrin signaling and via TGF- β	[25–27]
Angiogenesis	Endothelial cells Cancer cells	Apoptosis Inhibition of neovascularization Inhibits proliferation and migration Inverse correlation with VEGF expression	Via dephosphorylation and CD36 via FGF-2 via bFGF no known direct link	[28–33]
Immune system	T cells Tregs Macrophages NK cells Dendritic cells	Inhibits T-cell activation T-cell migration Induction of CD4+ Tregs Activation and recruitment Proliferation Affects maturation, trafficking, and anti-tumor responses	CD47 and integrin-associated protein heparan sulfate proteoglycans-mediated inhibition of TCR signaling. TSP-1 also inhibits H_2S-mediated MAPK signaling SNAIL-induced EMT leading to metastasis TLR-4 pathway, plasminogen activator inhibitor-1 Via TGF- β Via CD47	[34–45]

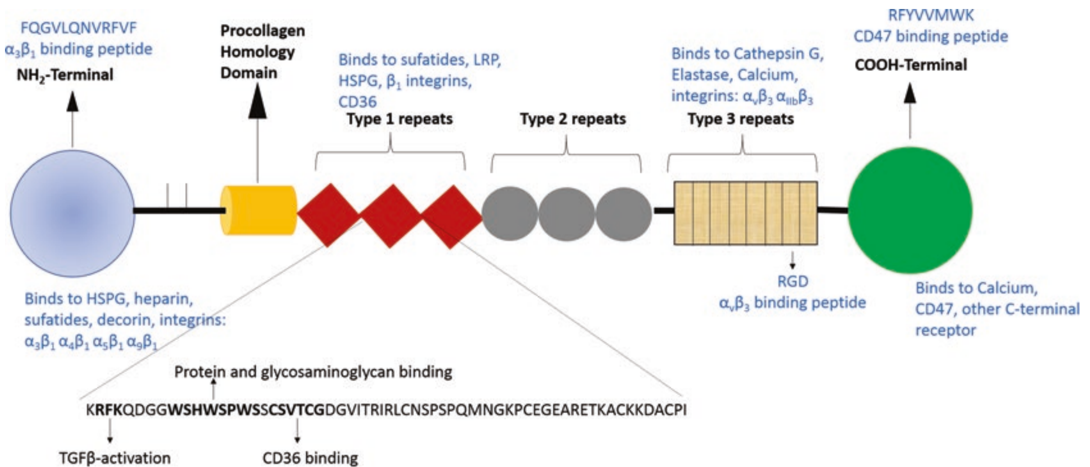

Fig. 8.1 Domain architecture of TSP-1 and TSP-2. Different domains and active peptide sequences that bind to different receptors are depicted

8.3.2 CD36 or GPIIIb

CD36 (cluster of differentiation 36) is a membrane glycoprotein expressed on the surface of a wide range of cells including platelets, monocytes, megakaryocytes, endothelial cells, mammary epithelial cells, and cancer cells. The binding of TSP to CD36 is facilitated through VTCG sequence in the TSRs of TSP. Interaction of TSP and CD36 has been implicated in various functional outcomes including angiogenesis inhibition [56, 57], activation of TGF-β1 [58], plate-

let aggregation [59], platelet-tumor cell adhesion [60], etc. Apoptosis of endothelial cells via TSP-1 is mediated through CD36 receptor by the activation of CD36-p59fyn-caspase 3-p38MAPK cascade, c-Jun N-terminal kinases, and Fas/Fas ligand [56, 57, 61].

8.3.3 Integrins

The integrin-binding RGD domain in TSP is located in the seventh type 3 calcium-binding repeat. Apart from binding to αvβ3 and αIIbβ3 on the platelet surface, TSP also binds to αvβ3, αIIbβ3, α3β1, α4β1, and α5β1 [62]. Integrin-mediated TSP functions include insulin-like growth factor signaling [63], neurite growth [64], phagocytosis [65], and cancer cell spreading via α3β1 [19].

8.3.4 Integrin-Associated Proteins (IAP or CD47) and Other C-Terminal Receptors

The two active peptides in the C-terminal of TSP that support cell attachment are RFYVVMWK and IRVVM [66, 67], which bind to the 52 kDa receptor CD47 [68]. IAP or CD47 has been associated with mediating cell adhesion properties of TSP. TSP-CD47 interaction affects integrin-mediated activities through modulation of αvβ3 on melanoma cells, αIIbβ3 on platelets, and α2β1 on vascular smooth muscle cells [69–71]. TSP-CD47 interaction reduces inflammation [72]. TSP bound to CD47 on T-cell surface induces the expression of BNIP3 (Bcl-2/adenovirus E1B 19 kDa-interacting protein) to mediate T-cell apoptosis and thus reduce inflammation [72]. Apart from calcium-dependent integrins and CD47, TSP also binds to another receptor termed M_r 80,000/105,000 at its C-terminus that has two component molecular weights: 80 kDa and 105 kDa [73]. This receptor binds to TSP in a Ca^{2+}- and Mg^{2+}-dependent fashion and is cross-reactive with members of the β1or β3 integrin receptor families [62, 73].

8.4 Functional Roles of Thrombospondin

8.4.1 Cell Adhesion and Motility

Interaction of TSP with various cell surface receptors, including integrins and sulfatides, is involved in mediating cellular attachment, spreading, and cell motility. The peptide sequence WSPW from the second type 1 repeat in TSP and the heparin-binding domain in the N-terminus promote endothelial cell adhesion while inhibiting basic fibroblast growth factor-mediated endothelial cell chemotaxis [74]. TSP-1 is known to mediate cell attachment in breast cancer, melanoma, small cell lung cancer, etc. via its interaction with its receptors including integrins and sulfatides [19, 20, 22, 75]. Contrary to this, TSP-1 type 1 repeats are also known to inhibit endothelial cell migration through β_1 integrins via a PI3k-dependent signaling [76]. Thus, TSP-1 may act differently depending on the receptor binding and downstream signaling.

8.4.2 Angiogenesis

TSP serves as a naturally occurring inhibitor of angiogenesis, which is critical for both tumor growth and metastasis. TSP negatively regulates angiogenesis either by inhibiting proliferation of endothelial cells [77] or by inducing their apoptosis [57]. Binding of TSP-1 to CD36 promotes downstream activation of $pp59^{fyn}$, p38MAPK, and caspase-3 to induce apoptosis in endothelial cells [57]. TSP-1 also inhibits lymphangiogenesis by binding to CD36 on monocytes and inhibiting lymphangiogenic factors: VEGF-C and VEGF-D [78]. TSP-1 inhibits the expression of platelet-endothelial cell adhesion molecule-1 in brain endothelial cells, causing altered cell-cell interactions and reduced formation of vascular networks [79]. However, a recent study demonstrated increased TSP-1 expression in the vasculature of angiogenic tumors [80].

8.4.3 Blood Flow

TSP-1 produced by vascular cells is present in the plasma and regulates blood flow to tissues. TSP-1 prevents NO/cGMP signaling to relax vascular smooth muscles, thereby regulating blood vessel tone and blood pressure to tissues [81]. Moreover, TSP-1 also blocks anti-thrombotic activity of NO/cGMP signaling axis [82]. These inhibitory responses require both TSP-1 receptors: CD36 and CD47 [83, 84]. Deficiency of either CD47 or TSP-1 alters the resting blood pressures and vasoactive stress responses [85]. Consistent with this notion, administration of a recombinant antiangiogenic domain of TSP-1 (three TSP-1 type 1 repeats or 3TSR) reduced tumor blood flow in an orthotopic model of pancreatic cancer [86]. TSP-1 overexpression by tumor has been found to moderately reduce tumor blood flow in response to vasoactive agents like NO or epinephrine in a melanoma model [87]. TSP-1 also alters blood flow to ischemic tissues via ROS production [88]. TSP-1-CD47 binding attenuates vasodilation and blood flow to injured tissues via activation of PKC and p47phox that promotes Nox1 to produce superoxide anions, which regulate vascular tone [88].

8.4.4 Activation of TGF-β

TGF-β is a multifaceted cytokine involved in a multitude of cellular functions including cell growth, differentiation, immune modulation, and inflammation. TGF-β activation requires TSP-1 central type 1 repeats containing amino acids KRFK. TSP-1 interacts with the LSKL sequence of the N-terminal domain of latency-associated peptide (LAP) of latent TGF-β, and instead of cleaving LAP for the activation (as seen with plasmin-mediated TGF-β activation) [89], TSP-1 induces a conformational change at this site to improve the accessibility of TGF-β to its receptor [90]. LAP with deleted LSKL is unable to bind or activate TSP-1 and thus is unable to provide latency to TGF-β [91]. The critical role of TSP-1 in promoting TGF-β activation has been demonstrated in pathologies including tumor growth and inflammation. TGF-β itself has been recently shown to increase TSP-1 expression in glioblastoma via SMAD3-dependent direct transcriptional activation [92]. Increased TSP-1 expression then leads to higher invasion through its interaction with CD47 receptor [92].

8.5 Thrombospondins in Carcinogenesis

Thrombospondins are both expressed by malignant cells and non-malignant cells in the tumor microenvironment [2]. In addition to angiogenesis modulation, the effects of TSP-1 on tumor progression are multifaceted and sometimes opposed, depending on the molecular and cellular interactions of its domains and cell surface molecules [17]. For instance, in melanoma, TSP-1 exhibits inhibitory effects of tumor cell migration (chemotaxis and haptotaxis) and proliferation. Inhibition of tumor cell proliferation was observed in both CD36-negative (more sensitive to TSP-1 effects) and CD36+ melanoma cells (less sensitive to TSP-1 inhibition), which is in contrast to the effects of TSP-1 on endothelial cells [93]. This anti-proliferative effect was ameliorated by inhibition of tyrosine kinase (phenotype reversed by herbimycin) or phosphatase (phenotype reversed by vanadate) [93]. One such tyrosine phosphatase receptor for TSP-1 was later found to be CD148 receptor-type protein tyrosine phosphatase, which negatively regulates cell growth upon TSP-1 binding [94]. TSP-1 deficiency reduces survival in the absence of p53 [95]. High and prolonged exposure of tumor cells to stromal TSP-1 has been found to lead to an increased breast cancer progression [96]. TSP-1 is also known to enhance invasion of oral squamous cell carcinoma cells by the upregulation of MMP9 through its interaction with integrin receptors [25]. As a tumor suppressor, TSP-1 null mice have been found to have larger mammary tumors, with higher METs and higher TGF-β expressions compared to wild-type mice [97]. TSP-1 induces monocyte-mediated killing of squamous carcinoma [98] and macrophage-mediated cytotoxicity of tumor cells [39, 40].

TSP2 is a more potent inhibitor of angiogenesis than TSP-1 and reduces tumor growth, even in the presence of high VEGF levels [15]. Thus, thrombospondins can act as both suppressors and promoters of carcinogenesis in the tumor microenvironment.

8.6 Thrombospondins in Tumor Microenvironment

The finding that TSP-1-deficient mice exhibit increased growth of implanted tumors provided direct evidence on the functional contribution of host TSP in carcinogenesis. In either homozygous or heterozygous p53 null mice, which develop spontaneous carcinomas, TSP-1 deletion reduced overall survival and increased progression of the disease and the associated mortality [95]. With numerous receptor-mediated interactions and varying expressions of TSPs in cancer, activities of TSPs in regulating different functions in the tumor microenvironment are detailed below.

8.6.1 Angiogenesis and Tumor Growth

TSP-1 and TSP-2 are both endogenous inhibitors of angiogenesis. Overexpression of TSP-1 inhibits its malignant tumor progression and metastasis associated with angiogenesis suppression [99]. Similarly, TSP-2 overexpression by fibroblasts can also inhibit growth of squamous cell carcinomas, malignant melanomas, and Lewis lung carcinomas by angiogenesis inhibition [100]. TSP-2 mediates endothelial cell apoptosis and inhibits the migration of endothelial cells to suppress neovascularization and, thus, tumor growth [101]. The antiangiogenic activity of TSP-1 has been mapped to two domains in its sequence: type 1 repeats (TSRs) and procollagen homology region [28]. This antiangiogenic activity of TSP-1 is not dependent on the presence of TGF-β-activating sequence (RFK), whereas the effect on tumor cell growth and apoptosis depends on TGF-β [9]. In a breast cancer model, deficiency of TSP-1 allowed formation of distended capillaries and sinusoidal vasculature, enhancing tumor growth and expression of VEGF, VEGFR2, and MMP-9. In contrast, TSP-1 overexpression delayed tumor growth associated with reduced vascularization [102]. TSP-1 also mediates antiangiogenic activity via endothelial cell apoptosis. Tumor cell-derived TSP-1 is critical for inducing cyclophosphamide-dependent endothelial cell apoptosis. Consistent with this finding, a lack of TSP-1 abrogates sensitivity of tumors to low-dose cyclophosphamide [103]. TSP-1 has opposite effects on endothelial cells and perivascular supporting cells (vascular smooth muscle cells and pericytes) [104]. TSP-1 along with platelet-derived growth factor (PDGF) and fibronectin induces chemotaxis of vascular smooth muscle cells, useful in response to arterial injury [105]. TSP-1 is very important in the proliferation and migration of pericytes during retinal vascular development [106]. This effect of TSP-1 on pericytes may have a paradoxical consequence as type 2 pericytes are involved in angiogenesis of ischemic tissues and may also improve blood flow in cancer [107].

The antiangiogenic activity of microenvironmental TSP-1 is necessary to inhibit tumor outgrowth. However, tumors generally overcome this inhibition to sustain their survival and maintain malignant progression. For example, Ras-mutant tumors inhibit fibroblast TSP-1 production via GPCR/SIP and Id1, in order to overcome the suppressive effects of TSP-1 [108]. Loss of p53 alleles leads to reduced TSP-1 secretion and increased VEGF secretion that facilitates malignant transformation [109]. Consistent with this observation, topical delivery of p53 to the lungs of mice reduced tumor burden in the lungs in a B16-F10 melanoma model [110]. Tumor suppression was associated with reduction in angiogenesis and VEGF production, while TSP-1 levels and mice survival increased [110]. Additionally, in the absence of active MYC, introduction of TSP-1 in p53 null tumors suppressed angiogenesis in order to regress tumor growth [111].

Stroma-derived TSP-1 has the potential to inhibit angiogenesis by suppressing NO signal-

ing via the inhibition of guanylate cyclase and cGMP-dependent protein kinase [81]. TSP-1 mediates its effects via both CD47 and CD36 receptors, where CD47 is more sensitive and also critical for the effects through CD36 [112]. These effects of TSP-1 may lead to a reduction of tissue perfusion of other organs and increased perfusion of the tumor as the vasodilation in tumors is less prone to the acute effects of NO signaling, thereby mediating TSP-1 side effects in tumor progression [112]. Prosaposin, a potent inhibitor of tumor metastasis, stimulates TSP-1 in monocytes, which via binding to CD36 receptors on the tumor cells mediates tumor suppression [113].

8.6.2 Cell Morphology, Adhesion, and Migration

TSP is an adhesive glycoprotein with multiple domains including N-terminal heparin-binding domain, COOH-terminal cell or platelet-binding domain, and RGDA sequence that mediate cellular adhesion, migration, and spreading [53, 54, 114–117]. CSVTCG in the type I repeats of TSP-1 promotes cell attachment [118]. A correlation between CSVCTG-specific TSP-1 receptor on gastric cancer cells and upregulation of MMP-9 has been associated with an aggressive tumor phenotype [119]. Expression of TSP-1 in mesangial cells has been found to be upregulated by signaling pathways involved in cell morphology and spreading. Src family kinases, ERK1/2 and small GTPase Rac-1, along with soluble factors in the serum upregulate the expression of TSP-1 in mesangial cells upon changes in cellular morphology [120]. TSP-1 is known to increase cancer cell invasion [121, 122]. Exogenous TSP-1 increases MMP-9 levels in endothelial cells, promoting their invasion and morphogenesis into tube-like structures [123]. Thrombospondin, via TGF-β, has been suggested to increase the expression of extracellular matrix-degrading proteases, urokinase plasminogen activator and plasminogen activator inhibitor-1, and a reduction in plasmin activity, leading to increased cell attachment, enhanced flattened cell morphology, and cell spreading, thus increasing cancer metastasis [24, 124–126]. Peptides from the type I repeats of TSP, which consists of the sequence Trp-Ser-Xaa-Trp, inhibit the binding of heparin and sulfatide to the amino terminus of TSP and promote cell adhesion of melanoma cells [127].

Different domains in TSP mediate tumor cell chemotaxis and haptotaxis. The carboxyl domain is responsible for TSP-induced haptotaxis, whereas NH2 heparin-binding domain of TSP affects only chemotaxis and not haptotaxis in cells [128].

8.6.3 Inflammation

TSP-1 expression is elevated during inflammatory responses. TSP-1 promotes the production of inflammatory cytokines IL-6, IL-1β, and TNF-α via NF-kB pathway in human monocytes, and inhibition of TSP-1 expression reduces cytokine production [129].

CD47 induces both T- and B-cell death to reduce inflammation [130, 131]. This clearance of lymphocytes is mediated by the binding of both TSP-1 and TSP-2 (and not SIRP ligands) to CD47 through their COOH-terminal domain. Proapoptotic signals in T cells mediate the translocation of CD47-bound BNIP3 to the mitochondria to trigger a mitochondrial cell death [132]. Deficiency of either TSP-1, TSP-2, or CD47 limits the clearance of activated T cells [72]. CD47-TSP-1 interaction is also responsible for the generation of CD4+ CD25+ Tregs from CD4+ CD25- naïve or memory T cells, which suppresses proliferation and cytokine production by Th0, Th1, and Th2 cells by a contact-dependent and TGF-β-independent mechanism to reduce inflammation [133]. A TGF-β-dependent mechanism of inflammation suppression by TSP-1 is also described. TSP-1-primed monocytes increase the expression of TGF-β, which induces Tregs to suppress inflammation [134]. In cancer, the role of TSP-1 is context dependent. For

example, in inflammation-induced cancers like colorectal cancer, TSP-1−/− mice show a lower tumor burden, despite an increase in new blood vessels and increased tumor cell proliferation, compared to the wild-type mice [135].

8.6.4 TSP in Metastasis Microenvironment

Malignancies including breast, skin, and colon cancer secrete factors and extracellular vesicles, which systematically reprogram distant organs including the lung to generate "pre-metastatic niches" (PN) [136]. Studies in mouse models have shown that these PNs characterized by host immune/inflammatory cells, organ-specific chemo-attractants, growth factors, and ECM-modifying proteins provide permissive microenvironments that support extravasation, colonization, and metastatic outgrowth of DTCs [136, 137]. The PN has become an exciting area of research in the quest for novel therapeutic and prophylactic strategies against metastasis [138, 139].

Primary tumor-derived TSP-1 has been shown to reduce the growth of spontaneous lung metastasis in melanoma patients and murine melanoma models [140]. In the absence of this tumor-derived TSP-1, lung metastasis had higher levels of neovascularization and tumor growth at secondary sites. In contrast, a novel mechanism was recently described, whereby metastasis-incompetent tumors generate metastasis-suppressive microenvironments in the lungs by inducing the expression of TSP-1, in the recruited bone marrow-derived myeloid cells [141]. TSP-1 induction was mediated by the activity of prosaposin (PSAP), a protein secreted by poorly metastatic cells, which acts systemically to reprogram myeloid cells into metastasis-inhibitory cells [141]. As another mechanism, inflammation in lungs led to the recruitment of bone marrow-derived neutrophils, which through the process of degranulation released Ser proteases: cathepsin G and elastase. These enzymes lead to the proteolytic degradation of TSP-1, causing metastatic outgrowth [142].

Following dissemination of primary tumor cells to distant organs, DTCs are not immediately competent to initiate growth and can persist in a dormant state [143]. Tumor recurrence occurs when dormant cells in metastatic niches escape their quiescent state. Thus, it is important to understand the mechanisms that keep tumor cell outgrowth at bay or even help in killing these dormant disseminated tumor cells in the microvasculature of different organs like the bone marrow, lungs, or brain. Endothelial cell-derived TSP-1 in the metastatic niche has been shown to induce DTC dormancy [144].

8.6.5 Tumor Immunity

Apart from its antiangiogenic potential, TSP-1 has the ability to reprogram immune microenvironment. Various immune and myeloid compartments are affected by TSP-1.

TSP-1 mediates complex effects on macrophages. TSP-1 via α6β1 enhances M1 macrophage differentiation and recruitment at tumor sites, promoting tumor cell death via superoxide production [39]. TSP-1-CD47 interaction inhibits IL-12 release by macrophages [145]. TSP-1 also inhibits LPS-induced IL-1β transcription in macrophages by limiting CD47-CD14 interaction [146]. Through CD36 receptor, TSP-1 mediates IL-10 production in macrophages [147] and partially mediates the activation of TLR-4 pathway in macrophages [38]. In cardiovascular physiology, TSP-1-CD47 interaction and NADPH oxidase 1 signaling are critical for the macropinocytic uptake of native LDL by macrophages [148]. Endothelial cells also increase TSP-1 levels via B-raf/MEK/ERK pathway to allow the recruitment of macrophages at these sites and enable clearance of apoptotic cells [40]. TSP-1 synthesized by monocytes is also known to play a role in the killing of undifferentiated squamous carcinoma cells [98]. TSP-1 null mice show symptoms of acute pneumonia with accumulation of neutrophils and macrophages in the lungs [149]. TSP-1 may serve as an important target to control obesity-induced inflammation, as TSP-1-deficient mice show reduced macro-

phage accumulation and inflammatory cytokine production than wild-type mice [150].

In dendritic cells (DCs), TSP-1 acts as an autocrine negative regulator [43]. Human iDCs produce TSP-1 which is enhanced by PGE2 and TGF-β secreted by phagocytic macrophages. This TSP-1 interacts with CD47 to reduce the secretion of IL-12, TNF-α, and IL-10, leading to a reduction in protective and inflammatory immune responses [43]. TSP-1 also is responsible for dysfunctional CD1a + MDDC (a DC subset differentiated from circulating human peripheral blood MO in response to specific inflammatory cytokines/pathogens) differentiation via activation of the inhibitory phosphatase, SHP-1 through CD47 receptor, leading to reduced T-cell stimulation and defective inflammatory immune responses [44]. CD47 is also critical for proper DC trafficking to elicit an immune response [42]. In support of the negative impact of TSP-1 on DC function, it was demonstrated that loss of TSP-1, but not TSP-2 in DCs, increased anti-tumor immune responses by increasing infiltration of CD4+ and CD8+ T lymphocytes and enhanced the production of IL-12 and IFN-γ, resulting in delayed tumor growth [45]. TSP-1 also mediates a negative impact on NK cells. TSP inhibits early NK cell proliferation but stimulates late NK cell expansion in a TGF-β-dependent fashion [41].

TSP-1 has been implicated in immunosuppressive responses via induction of Tregs [151] and possibly TGF-β [152]. In a melanoma model of EMT, Snail promotes immunosuppressive effects by inducing Tregs and impairing dendritic cells, expressing high levels of IDO via TSP-1 production. Inhibition of either Snail or TSP-1 increased systemic immune responses, associated with decreased tumor growth and metastasis [37]. These immune responses were found to be independent of TGF-β. However, TGF-β2-expressing antigen-presenting cells require TSP-1 in the microenvironment in order to induce FOXp3+ Tregs both in vitro and in vivo [153].

TSP-1 is an endogenous inhibitor of T-cell activation and differentiation via its interaction with CD47 receptor. TSP-1 inhibits hydrogen sulfide-mediated MEK/ERK signaling in T cells for their activation. TSP-1 also inhibits autocrine activation of T cells by hydrogen sulfide via downregulation of cystathionine β-synthase and cystathionine γ-lyase enzymes (biosynthetic H_2S enzymes) in T cells [35].

8.7 Future Commentary

Thrombospondins affect cellular functions by autocrine, paracrine, and indirect mechanisms that may involve interaction with proteins like TGF-β and lead to tissue remodeling, endothelial cell behavior, and cancer suppression/progression. Secreted by both tumor cells and the stroma, TSPs can mediate both suppressive and cancer-promoting behaviors. Cancer-promoting roles may warrant inhibition of TSPs as a therapeutic modality to suppress tumor growth/progression. Therapeutic strategies targeting TSP-1 are already being pursued which include either TSP-mimetic peptides, antibody blockades, or strategies to upregulate endogenous TSPs. Compounds like ABT-510 (TSR1s) and CVX-045 (TSP-1 mimetic) have been in Phase II and Phase I clinical trials, respectively [17]. ABT-510 was discontinued from clinical trials due to its low objective response rate and 1-hour half-life [17]. Although CVX-045 demonstrated efficacy in combination with chemotherapy, severe adverse events (radiation pneumonitis and bowel obstruction with perforation leading to death) associated with CVX-045 led to discontinuation of its further development [154]. ABT-898, a second-generation TSP-1 mimetic peptide with increased stability and reduced toxicities, has been indicated for inhibiting ovarian cancer progression. Trabectedin, indicated for advanced soft tissue sarcoma and ovarian cancer, acts via upregulating TSP-1. An increase in TSP-1 via prosaposin (psap) provides a suitable translational potential as an anti-metastatic therapy. Psap, a five-amino acid peptide, which is known to stimulate stromal p53 and TSP-1, has been shown to be a potent anti-metastatic agent in various cancer models [113, 141, 155]. Administration of psap causes production and

release of TSP-1 by monocytes present in the pre-metastatic niche, which then prevents metastatic colonization [113].

With multiple receptors binding to TSPs, there are numerous signaling pathways activated downstream which regulate complex dynamic processes in both normal physiology and pathology of a disease. Understanding these intercalating networks that act through TSPs is essential in developing the appropriate therapeutic strategy, which could have impact on the tumor microenvironment to suppress tumor progression using one of the cellular activities (angiogenesis, tumor immunity, etc.) affected by thrombospondins.

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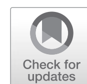

9 Tenascin-C Function in Glioma: Immunomodulation and Beyond

Fatih Yalcin, Omar Dzaye, and Shuli Xia

Abstract

First identified in the 1980s, tenascin-C (TNC) is a multi-domain extracellular matrix glycoprotein abundantly expressed during the development of multicellular organisms. TNC level is undetectable in most adult tissues but rapidly and transiently induced by a handful of pro-inflammatory cytokines in a variety of pathological conditions including infection, inflammation, fibrosis, and wound healing. Persistent TNC expression is associated with chronic inflammation and many malignancies, including glioma. By interacting with its receptor integrin and a myriad of other binding partners, TNC elicits context- and cell type-dependent function to regulate cell adhesion, migration, proliferation, and angiogenesis. TNC operates as an endogenous activator of toll-like receptor 4 and promotes inflammatory response by inducing the expression of multiple pro-inflammatory factors in innate immune cells such as microglia and macrophages. In addition, TNC drives macrophage differentiation and polarization predominantly towards an M1-like phenotype. In contrast, TNC shows immunosuppressive function in T cells. In glioma, TNC is expressed by tumor cells and stromal cells; high expression of TNC is correlated with tumor progression and poor prognosis. Besides promoting glioma invasion and angiogenesis, TNC has been found to affect the morphology and function of tumor-associated microglia/macrophages in glioma. Clinically, TNC can serve as a biomarker for tumor progression; and TNC antibodies have been utilized as an adjuvant agent to deliver anti-tumor drugs to target glioma. A better mechanistic understanding of how TNC impacts innate and adaptive immunity during tumorigenesis and tumor progression will open new therapeutic avenues to treat brain tumors and other malignancies.

Keywords

Tenascin-C · Extracellular matrix · Brain · Glioma · Tumor microenvironment · Integrin · Toll-like receptor · Adhesion · Proliferation · Angiogenesis · Cancer stem cells

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· Immunomodulation · Inflammation · Tumor-associated microglia/macrophages · T cells

Abbreviations

CNS	Central nervous system
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FBG	Fibrinogen-like globe
FGF	Fibroblast growth factor
FNIII	Fibronectin type III-like domains
GBM	Glioblastoma
GSCs	GBM cancer stem cells
HA	Hyaluronic acid
IL	Interleukin
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinases
PDGF	Platelet-derived growth factor
PRR	Pattern recognition receptors
TAMs	Tumor-associated microglia/macrophages
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TME	Tumor microenvironment
TNC	Tenascin-C
TNFα	Tumor necrosis factor-α

9.1 Introduction

Tenascin-C (TNC) is a multifunctional extracellular matrix (ECM) glycoprotein with distinct spatial and temporal expression patterns during embryonic development, tissue homeostasis, and disease, including various forms of solid malignancies. Over the last several decades, many reports have shown how TNC modulates cell adhesion, migration, proliferation, and angiogenesis in a context- and cell type-specific manner. However, little is known about its immunomodulatory function in pathologies, including malignant tumors of the central nervous system (CNS). In the following chapter, we are going to briefly discuss TNC structure, receptors/interaction partners, expression, and regulation in general, with an emphasis on brain tumors. We will focus on TNC function in cell-specific immunomodulation and discuss current therapeutic interventions as well as future directions.

9.2 Structure of Tenascin-C

TNC is the first member of the tenascin family discovered by various groups in the 1980s [34]. Other members of the family, tenascin-W, tenascin-X, tenascin-R, and tenascin-Y, show structural similarity with TNC and overlap with TNC function during development, wound healing, tissue remodeling, and diseases [94]. Since most discoveries and functional studies have been related to TNC, in this chapter, we will only focus on TNC.

The TNC protein consists of six subunits linked by bisulfide bonds; each subunit is ~250 kDa and has four distinct domains: a cysteine-rich amino terminus with heptad repeats, 14.5 epidermal growth factor (EGF)-like repeats, eight constitutive fibronectin type III-like (FNIII) domains, and a C-terminal fibrinogen-like globe (FBG) [94]. These motifs generate a highly versatile structure, due to which TNC has the capacity to interact with multiple proteins [131]. For an illustration of TNC domains, please refer to many other reviews [94, 155].

Contributing to the multi-modular structure of TNC is its expression in several variants [35], which are due to alternative splicing and post-translational modifications, such as glycosylation. Western blotting analysis of the TNC protein from human brain tumor cells showed multiple bands between 250 and 300 kDa [204]. In human TNC, there are eight FNIII repeats (FNIII 1–8) constitutively presented. Between the fifth and sixth FNIII domain, a series of FNIII domains (up to nine) may be presented, due to alternative splicing in a varying fashion [131]. These highly heterogeneous FNIII domains of TNC can generate more than a cou-

ple of hundred potential isoforms that differ in proteolytic cleavage sites and post-translational modifications [59].

9.3 Tenascin-C Receptors and Interaction Partners

TNC's highly versatile structure allows it to interact with various ligands, for example, the ECM itself [39], integrins [214], pattern recognition receptors (PRR) [226], and soluble factors [44]. The most well-studied ECM binding partner of TNC is fibronectin, which interacts with TNC via the binding sites located throughout the FNIII repeats of TNC [39]. Other ECM-related molecules that bind to TNC are collagens [135], periostin [104], SMOC1 [18], fibrillin-2 [19], and proteoglycans of the lectin family [7]. The capability to bind ECM components suggests that TNC might play a role in the structural organization of the tumor microenvironment; evidence of such mechanisms mediated by TNC already exists in brain tumor [20]. In addition, TNC has been shown to affect ECM stiffness and mechanosignaling in various tissues [88]. TNC also binds to syndecan-4 [173], glypican [2], and receptor-like protein tyrosine phosphatase beta zeta (RPTPβ/ζ) [4], among a few other membrane-binding proteins. For a detailed description of TNC binding sites to these partners, please see Midwood et al. [132].

The interactions between integrins and the ECM molecules play an important role in cancer biology by propagating metastasis and invasion [68]. Seven integrins have already been shown to directly bind to TNC FNIII and FBG domains, including α9β1, αvβ3, α8β1, αvβ6, α2β1, a7β1, α7β1, and α8β1 [196]. The general function of these TNC-integrin interactions in tumors is associated with epithelial-mesenchymal transition (EMT), migration, and poor patient survival [214].

TNC takes part in innate immunity by directly binding to toll-like receptor 4 (TLR4) on immune cells [130] or pathogens, e.g., the human immunodeficiency virus (HIV) [54]. The EGF-like repeats of TNC have been shown to bind directly to the EGF receptor (EGFR) and promote cell proliferation [191].

A growing number of TNC interaction partners are soluble factors [44]. These consist of a wide spectrum of growth factors, which include members in the platelet-derived growth factor (PDGF) family, the fibroblast growth factor (FGF) family, the transforming growth factor-beta (TGF-β) superfamily, the insulin-like growth factor binding proteins, and neurotrophins. All these interactions are mediated by TNC FNIII 4–5 subdomain [44, 126]. TNC has also been described to promote Wnt/β-catenin signaling by binding to Wnt3a in the whisker follicle stem cell niches [78] and injured kidney [28].

9.4 Expression of Tenascin-C in Glioma

Unlike the periphery system, in which the main ECM components are fibrillar collagen, fibronectin, laminin, etc., the ECM of the CNS is enriched with various non-fibrillar components such as proteoglycans and glycoproteins [163]. Together with thrombospondin-1 (TSP-1) and SPARC, TNC belongs to the matricellular proteins, which are secreted and rapidly turned over molecules in the ECM with mainly regulatory functions instead of structural supportive roles [16]. In contrast to other ECM components in the CNS, e.g., the abundant expressed hyaluronic acid (HA), TNC is only highly expressed during normal fetal development [137]. In support of TNC expression in the developing brain, persistent levels of TNC have been characterized in adult neural stem cell niches, including the radial glia and astrocyte stem cell compartment of the subventricular zone (SVZ) [50]. Other parts of adult brains have an undetectable level of TNC, and persistent TNC expression is associated with a variety of chronic neuropathological conditions, including brain cancer.

Grade IV glioma, glioblastoma (GBM), is the most common and malignant primary brain tumor in adults and comprises ~25,000 new cases annually in the United States [182]. The median survival of patients diagnosed with GBM is less

than 2 years. The ECM in the glioma microenvironment is often deregulated [197], and abnormalities in the ECM affect cancer progression by directly promoting cellular transformation, tumor cell migration, invasion, tumor-associated angiogenesis, and tumor-associated inflammation [51].

TNC is abundantly expressed in GBM [25] as well as multiple other solid malignancies, including breast cancer [17], prostatic adenocarcinoma [102], and colorectal carcinoma [73]. In solid tumors of the periphery system, fibroblasts in the tumor stroma are a major source of TNC deposited in the tumor microenvironment (TME) [70, 82]. In contrast, in GBM, malignant tumor cells are the main source of TNC production [202]. Besides neoplastic cells, endothelial cells have also been found to be a source of TNC in the CNS [21, 125]. Complimentary studies establish the prognostic value of TNC as a biomarker for poor patient survival and disease progression [41, 61, 114, 148, 162, 210–212]. In glioma, TNC has been established to correlate with its respective grade [105]. In TCGA, data of low-grade glioma (LGG) and high-grade glioma (HGG) display a significant difference in survival between patients with TNC-high (median = 25.2 months) and TNC-low (median = 95.6 months) expression (Fig. 9.1).

Many solid tumors, including GBM, exhibit cellular heterogeneity and differentiation hierarchy in which GBM cancer stem cells (GSCs) are at the apex [186]. GSCs exhibit the capacity of self-renewal, multi-lineage differentiation, and tumor initiation [56, 213]. Multiple studies, including mass spectrometry and lectin-microarrays [76, 145], established TNC as a specific cell surface marker for GSCs. In Xia et al., immunofluorescence staining of TNC expression in orthotopic xenografts derived from GSCs showed extensive and strong TNC expression in the extracellular space of brain tumors [204].

The perivascular niche is a well-established compartment of the tumor microenvironment, known for its enrichment of GSCs [23]. Broad expression analysis of TNC in human gliomas of varying grades revealed the association of TNC in this particular niche, and TNC level in the perivascular niche correlated with angiogenesis and a shorter disease-free time [79, 217].

As mentioned earlier, depending on its transcriptional and post-transcriptional modifications, TNC appears in a variety of isoforms [35]. Characterization of specific TNC isoforms in the tumor microenvironment has shown that the large isoform of TNC is abundantly expressed in multiple solid malignancies, including GBM [17, 25, 73, 102]. Monoclonal antibodies (mAb) designed to interact with specific epitopes of TNC large isoforms displayed three distinct staining patterns in human glioma [21]: the mAbs stained

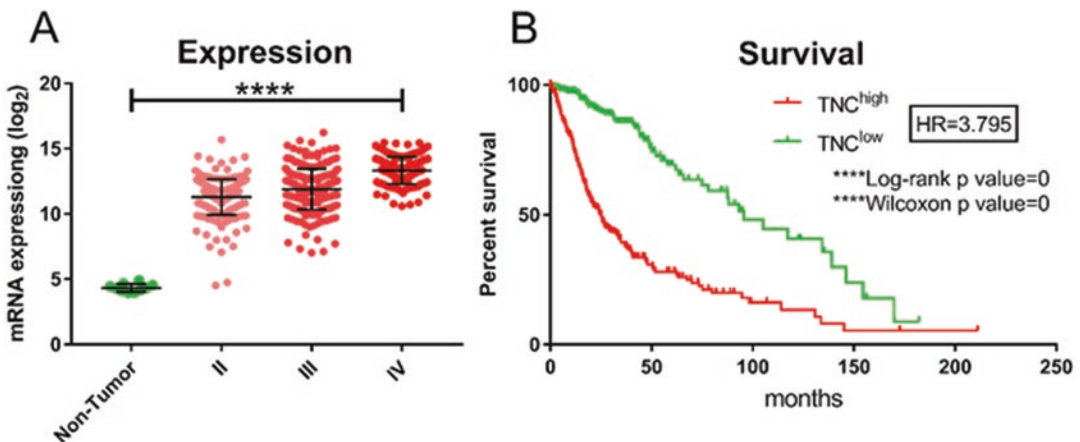

Fig. 9.1 (**a**) Expression of TNC in non-tumor tissues ($n = 10$) and grade II ($n = 226$), III ($n = 244$), and IV gliomas ($n = 150$). (**b**) Kaplan-Meier survival curve in TNC^{high} ($n = 334$) versus TNC^{low} ($n = 336$) patients. Cutoff, median; ****, p-value = <0.0001; error bar represents standard deviation (SD)

stroma, the TNC-enriched individual cells, and the perivascular niche. While the stromal and perivascular patterns of TNC expression have already been reported [79], the authors hypothesized that the TNC-enriched cells might be representative of TNC⁺-GSCs. A crucial part of this study was, however, that these patterns were stained in varying intensity with the mAb, displaying the complexity of TNC isoform expression in glioma [21]. Overall, these studies suggest that TNC, resurfacing in glioma, is associated with its role and function during neural development and tumor progression.

9.5 Regulation of Tenascin-C

TNC is a very strictly regulated ECM component, as it is highly expressed during fetal development but almost undetectable in most adult tissues. TNC is rapidly and transiently induced in acute pathologies in a stimulus-based manner and persistently expressed in prolonged pathological conditions such as chronic inflammation and cancer. The TNC gene is located on chromosome 9q33 and consists of 29 exons [147, 222]; at its first exon, there is a transcription start site (TSS) with an upstream TATA box.

Upstream Signaling to Regulate TNC The regulation of TNC expression has been characterized in the context of fetal development, integrin-mediated signaling in mechanical stress, inflammation, tissue repair, and tumor progression [59]. For instance, during tissue repair, a fair amount of pro-inflammatory and anti-inflammatory cytokines have been reported to induce TNC expression, including IL-1α [124], IL-1β [29], IL-4 [64], and IL-13 [93]. Growth factors also play an indispensable role during tissue repair and have been described to regulate TNC gene expression [91, 92]. These regulators of TNC expression are also found in the tumor microenvironment, as inflammation and growth factor-mediated proliferation are hallmarks of cancer [69]. However, due to the huge amount of signaling pathways mediated by these cytokines and growth factors, it is still not very well-known which pathway specially regulates TNC expression. Furthermore, activation of different TLRs, including TLR4, has been shown to positively regulate TNC expression via PI3K/AKT- and NF-kB-mediated signaling pathways in monocyte-derived dendritic cells [62]. There are various other NF-kB upstream signals that can induce TNC expression, such as tumor necrosis factor-α (TNF-α) in human chondrocytes [143] and stress-induced reactive oxygen species (ROS) in rat cardiac myocytes [208]. In accordance with increased TNC expression during inflammation, glucocorticoids, which act as potent anti-inflammatory steroid hormones [42], suppressed TNC expression in bone marrow stromal cells and in fibroblasts [48].

Transcription Factors Cytokines and growth factors activate signaling transduction pathways that converge to transcription factors to regulate TNC expression. In human fibroblasts and astrocytes, TGF-β/Smad-signaling and FGF could induce the expression of TNC. Smad-mediated TNC upregulation required the formation of a complex together with co-factors, such as SP1, ETS1, and CBP/p300 [91]. A follow-up study identified PDGF as a regulator of TNC gene expression via the PI3K/AKT signaling [92]. In chicken embryo fibroblasts, these growth factors displayed even a synergistic effect to induce TNC mRNA expression [37].

C-Jun is a transcription factor that regulates TNC transcription. Various important cellular processes, e.g., proliferation, apoptosis, and survival, are regulated by C-Jun, alone or together with activator protein-1 (AP-1); and stimulus-based overactivation of C-Jun has been linked to tumorigenesis [128]. In Ras-induced oncogenic transformation of fibroblasts, TNC was upregulated via c-Jun, suggesting a role of TNC in cellular transformation [129]. C-Jun is not the only known transcription factor to regulate TNC expression in tumors. TNC was identified as a direct target gene for SOX4 [176], which is overexpressed in many human cancers, including glioma ([27]; [115]).

TNC and Notch Notch-signaling plays a crucial role in the development of a multitude of organs [200] by regulating cell-to-cell interaction and cell fate. In gliomas, Notch1 contributed to Ras-induced transformation of glial cells and glioma growth [98]. Tumor tissue microarray revealed the co-expression of TNC and RBPJk in glioma [188]; the latter is a Notch2 co-factor for transcription. The same study also revealed an RBPJk-responsive element in the TNC promoter and a Notch-mediated increase in TNC production. In human GSCs, TNC was found downregulated during GSC differentiation induced by retinoic acid (RA) [213]; the same study also identified the downregulation of Notch signaling following GSC differentiation. Recently, Ma et al. reported that TNC was dramatically upregulated in human GBM cells and preclinical GBM models following CD47 knockdown in GBM cells [119], a cell surface protein and a "don't eat me signal" that inhibits phagocytosis of tumor cells by macrophages [185]. A Notch-dependent mechanism of TNC upregulation following CD47 knockdown was proposed in the study, consistent with the works from Sivasankaran et al. [188] showing that TNC is a downstream target of Notch signaling in glioma. Interestingly, Sarkar et al. identified TNC as an important regulator to activate Notch signaling in GSCs [174], suggesting a positive feedback relationship between TNC and the Notch pathway in GBM.

Other Regulators in GBM Besides being downregulated during GSC differentiation, TNC was reduced in brain tumors and astrocytes carrying heterozygous IDH1 R132R mutation [201], possibly via a HIF-a-mediated mechanism. IDH1 is a metabolic enzyme, and its mutation is one of the early events during gliomagenesis and has been identified in 70–80% grade II/III gliomas and 10% GBM [209]. Interestingly, mutations in IDH1 are associated with a better prognosis and clinical outcome [224]. Oyinlade et al. reported that TNC was decreased when the synthesis of glycosaminoglycans in GBM cells was halted by knocking down UDP-glucose 6-dehydrogenase (UGDH) [152]. UGDH is the rate-limiting enzyme for the synthesis of the abundant ECM components in the brain, e.g., HA and a dozen of proteoglycans [47]. This report is consistent with the notion that TNC serves as an organizer of the matrix itself and provides a linker for many glycosaminoglycans in the ECM [207].

9.6 Functions of Tenascin-C

Numerous literatures support the current consensus of TNC functional dichotomy, a feature that can be attributed to different cell types, different disease stages and conditions, the multi-domain and highly interactive structure of TNC, various isoforms of TNC due to alternative splicing, and numerous TNC interacting receptors/partners. More importantly, some discrepancy and even seemingly contradictory findings in TNC function studies may stem from distinct experimental conditions in individual studies: exogenous TNC as a soluble factor versus TNC as an ECM component when used as a surface-coating agent and TNC knockdown in specific cell types versus TNC knockout in whole animals, to name a few. Nevertheless, the versatile properties and features of TNC allow a wide variety of effector mechanisms, which play into the crucial role of TNC in numerous pathologies, including GBM. In the following, we will go into these mechanisms in the context of tumor progression. Figure 9.2 demonstrates the overall impact of TNC on malignant tumors.

9.6.1 Cell Adhesion/Migration

The effect of TNC on cell adhesion has been extensively reviewed elsewhere [36, 59], and here we will briefly summarize major findings. Generally speaking, TNC modulates cell adhesion/ spreading in a context- and cell type-specific manner with both anti- and pro-adhesion activities. The large isoform of TNC has been shown to antagonize cell adhesion (or spreading) on fibronectin-coated surfaces. Thus, when com-

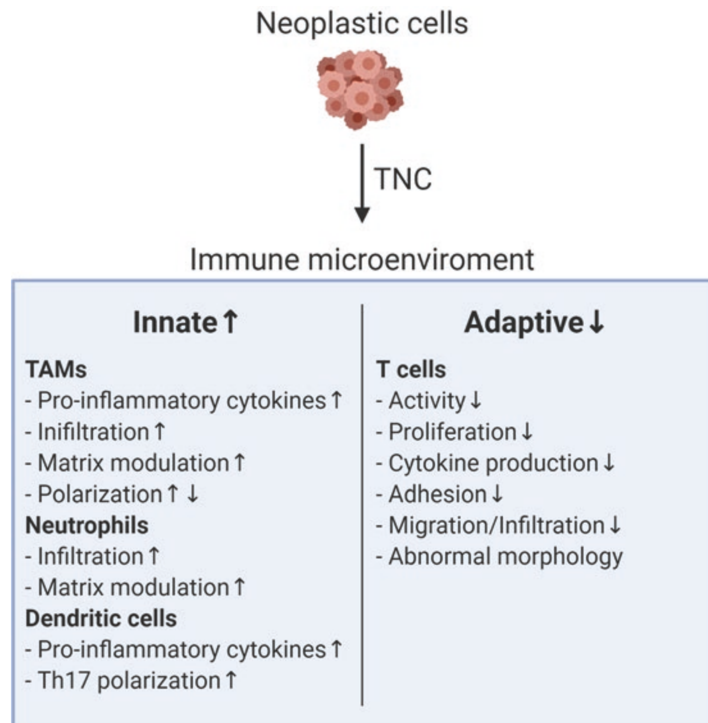

Fig. 9.2 Overview of the functions of tenascin-C in the tumor microenvironment of glioma. This figure is created with BioRender.com

pared with fibronectin, laminin, and many other ECM components, TNC has an anti-adhesive effect [31, 33]. In contrast, some small TNC isoforms bind strongly to fibronectin and promote focal adhesion and cell attachment [223]. TNC also promoted cell adhesion when used as a surface-coating molecule when compared to non-coated surfaces [52]. Translating this cell adhesion/anti-adhesion phenomenon to cell migration properties requires careful evaluation of experimental conditions as a fine balance between cell adhesion and detachment determines cell migration: cells need to adhere to the ECM to be able to migrate, yet too strong adhesion will reduce cell motility. In other words, an intermediate state of adhesion is favorable for cell migration [139].

Various reports from independent groups have consistently demonstrated that TNC positively modulates an infiltrative phenotype of glioma [51]. TNC-deficient GBM cells showed decreased invasion and alternated peritumoral ECM composition in murine glioma models [83]. TNC promoted the migration of human glioma cells by affecting their cellular response through fibronectin [45]. Additionally, TNC decreased focal adhesion formation and cell adhesion by modulating the focal adhesion kinase (FAK)/RhoA axis [133] and regulating integrin α5β1 through syndecan-4 [173], thus increasing motility. Besides modulating the ECM, inducing cell motility, and inhibiting focal adhesion, TNC also induced matrix destructing enzymes, such as matrix metalloproteinases (MMPs), to promote tumor cell migration [96]. Xia et al. [204] reported that knocking down TNC in GBM neurosphere cells enriched for GSCs increased cell adhesion on laminin-coated surfaces and decreased GBM cell migration via an FAK-dependent mechanism. In accordance with these in vitro findings, TNC knockdown dramatically decreased GBM cell migration in orthotopic xenografts derived from these GBM neurosphere cells. The same group also reported that increased TNC level was associated with an invasive tumor phenotype and irregular tumor border when CD47 was knocked out in GBM cells [119].

9.6.2 Angiogenesis

High abnormal vascularization defines GBM and contributes to tumor progression and their massive mortality rate [150]. As previously mentioned, several studies identified the expression of TNC in the perivascular niche [79, 113, 217]. In GBM, perivascular TNC has been correlated with vascular hyperplasia [79], increased vascular density [216], and elevated level of vascular endothelial growth factor (VEGF) [217]. Proteomic and immunohistochemistry analysis of angiogenesis in normal brains and in GBMs showed pathological TNC upregulation, as represented by endometrial blood vessels [141]. In a TNC knockout model of melanoma, TNC deficiency resulted in impaired tumorigenesis associated with a disrupted capillary net system. This effect was mediated by reduced expression of VEGF, displaying TNC's potential to modulate angiogenic factors [150]. In xenografts derived from GBM neurospheres, TNC knockdown was associated with decreased blood vessel numbers but increased blood vessel size [204]. Even though most studies positively linked TNC to angiogenesis, Rupp et al. demonstrated the dual function of TNC in GBM angiogenesis [171]: direct contact of endothelial cells with TNC downregulated YAP-dependent pro-angiogenic targets and consequently reduced endothelial cell proliferation, survival, and tube formation. On the other hand, GBM cells exposed to TNC induced a pro-angiogenic paracrine secretome and increased endothelial cell survival by upregulating ephrin-B2. This further speaks the cell-type specific function of TNC.

In the CNS, pericytes, endothelial cells, and astrocytes form the blood-brain barrier (BBB) [43]. In glioma, pericytes migrate into the tumor microenvironment and contribute to malignancy by promoting vascularization [15]. Differentiated pericytes displayed significant levels of TNC, suggesting not only a possible role of pericytes in vessel formation but also pericytes as another source of TNC in the brain tumor microenvironment [95, 179].

9.6.3 Proliferation

Exogenous TNC has been shown to increase brain tumor cell proliferation in vitro, and elevated TNC correlates with proliferation in human GBM [11]. In accordance with its angiogenic properties, TNC induces the proliferation of endothelial cells [24]. The mechanism of TNC-mediated cell proliferation is still not clear. Huang et al. reported that TNC prevented cell attachment and promoted cell proliferation by impairing cell attachment [86]. Thus, TNC-mediated cell proliferation may be explained by its effect on cell adhesion since cell proliferation and attachment are two closely related cellular phenotypes. That means integrins and their co-receptors are the main underlying mechanisms of the pro-proliferative function of TNC [215]. Another possible mechanism might be the capability of TNC to bind to EGFR [191].

However, much like TNC's effect on cell adhesion, TNC can exert both pro- and anti-proliferation functions in a context-specific manner. In GBM and breast cancer cells, binding of TNC to fibronectin interfered with syndecan-4-binding sites, resulting in a cell adhesion defect and concomitantly increased cell proliferation [86]. On the contrary, TNC was shown to block the cell cycle progression of anchorage-dependent fibroblasts on fibronectin through inhibition of syndecan-4 [151]. Xia et al. reported that in GBM cells, TNC knockdown had no effect on cell proliferation in vitro but increased tumor growth in vivo [204]. An alteration in tumor cell-immune cell interactions in response to TNC knockdown may contribute to the increased tumor growth following TNC downregulation in GBM cells, which will be discussed later.

9.6.4 Stem Cell Maintenance

The stem cell niche is a specialized environment that is able to sustain the undifferentiated state of stem cells [175]. The abundance of TNC in neural stem cell niche SVZ during development and

in adulthood suggests an important function of TNC in neural stem cell maintenance. Collective evidence indicates that TNC promotes neural stem cell proliferation and migration and inhibits their differentiation (maturation) [198]. Direct evidence of TNC modulating neural stem cell biology came from the observation that TNC knockout mice had decreased numbers of neural stem cells, which exhibit an increased capacity to differentiate into neurons in cultures [57]. Subsequent studies confirmed that TNC regulated neural stem cell migration and differentiation during development [50]. TNC-deficient mice exhibited increased maturation and suppressed proliferation of oligodendrocyte precursors [57, 58]; these mice also displayed a higher amount of immature astrocytes in the spinal cord without any changes of the white matter, proposing defects of migration [101]. Consistent with the role of TNC in stem cell maintenance, ExonArray analysis identified TNC as one of the top genes significantly downregulated during retinoic acid-induced GSC differentiation [213]. These attributes of TNC also translated into an aggressive phenotype of gliomas derived from these GSCs [204].

9.7 Immunomodulation

Although TNC is highly expressed during embryonic development, TNC deficient mice were born normally, suggesting that TNC is dispensable for normal development [172]. TNC is prominently re-expressed at sites of tissue renewal, remodeling, and regeneration, suggesting an important function of TNC when tissue homeostasis is disturbed, such as during infections, tissue injury, and cancer [32]. Indeed, TNC deficient mice showed altered inflammatory cytokine production in injured brains [87]. Since numerous innate and adaptive immune cells reside in tissue environments are involved in infection, inflammation, wound repair, and tumor progression, it is not unanticipated to uncover the role of TNC in immunomodulation [130]. In mouse models of Alzheimer's disease, asthma, cancer, liver and lung fibrosis, multiple sclerosis, myocardial infarction, and rheumatoid arthritis, TNC has repetitively been shown to contribute to fibrosis and inflammation [127].

More importantly, cells of the immune system express binding partners for TNC, including toll-like receptor 4 and integrins. Upon binding to these receptors, TNC promotes the expression of pro-inflammatory cytokines, including TNF-α, IL6, IL8, and many other cytokines, which in turn regulate TNC expression to form a positive feedback loop to influence a wide variety of immune cells [62, 130]. In fact, TNC has been recognized as an endogenous danger-associated molecular pattern (DAMP) that interacts with toll-like receptors and others to modulate the immune system [226]. A detailed list of these immune cell types and cytokines can be found in the excellent review by Marzeda et al. [127].

In the tumor immune microenvironment, TNC impacts a broad spectrum of immune cells such as macrophages, microglia, T cells, neutrophil, dendritic cell, and natural killer cells. In the following section, we are going to discuss how TNC affects each immune cell type and regulates immune response to influence tumor growth. A summary of these functions can be found in Fig. 9.3.

9.7.1 Tenascin-C Function in Macrophages and Microglia

Recruitment The microenvironment of GBM consists of brain-resident microglia and macrophages recruited from periphery blood. These so-called tumor-associated macrophages/microglia (TAMs) make ~30–50% of a tumor mass and play an important role in driving cancer formation, progression, and treatment resistance by creating an immunosuppressive and tumor-promoting microenvironment [67]. Neoplastic cells secrete various factors to induce the infiltration of blood-derived macrophages. Various studies have linked components of the ECMs, including HA ([109]; [149]; [195]; [220]), versican ([46]; [84]), and TNC ([119]; [144]; [193];

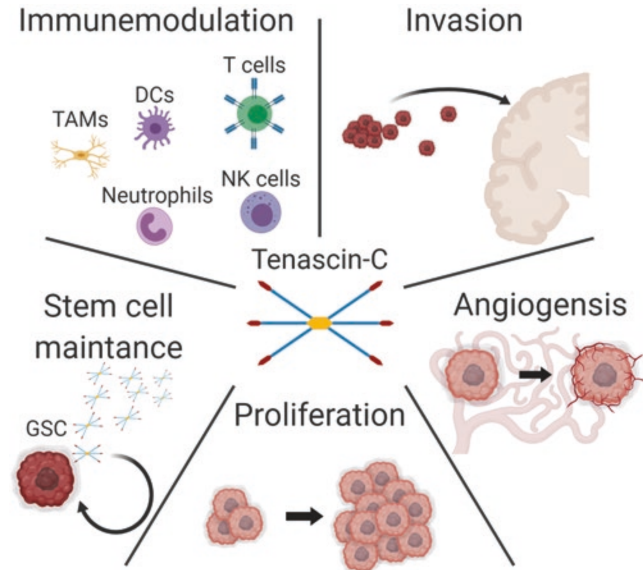

Fig. 9.3 Cell-specific modulation of immune cell activity through tenascin-C in the tumor immune microenvironment. This figure is created with BioRender.com

[212]), to facilitate TAM infiltration and their immunosuppressive function, partially because ECMs as structure proteins have the ability to immobilize growth factors and cytokine/chemokines, and serve as a storage depot for these factors to increase their local concentration to elicit biological impacts on immune cells.

Polarization and Pro-inflammatory Cytokine Release TAMs are highly heterogeneous, and their activation has traditionally been characterized as the M1 "classical" and M2 "alternative" polarization based on in vitro maturation and activation protocols. For example, lipopolysaccharide (LPS) and/or interferon-gamma (IFNγ) can induce the M1 polarization of human and murine macrophages, which upregulates surface marker CD80/CD86 and promotes the release of pro-inflammatory cytokines, such as TNF-α and IL-12. M1 macrophages can attack tumor cells by phagocytosis, producing pro-inflammatory cytokines and releasing nitric oxide (NO). M2 macrophages can be induced by interleukin-4 (IL-4) stimulation in vitro with an upregulation of macrophage mannose receptor (MMR, CD206) and scavenger receptor CD163, accompanied by an increase in anti-inflammatory and immunosuppressive cytokines IL10 and TGF- β. This M2 type of macrophage polarization is associated with pro-tumoral functions and usually upregulated during tissue repair, matrix remodeling, and immunosuppression.

The M1/M2 classification of TAMs is nowadays considered an oversimplified term [140]. For example, M2 macrophages can be further divided into M2a, M2b, M2c, and M2d subtypes based on distinct stimuli in vitro; each subtype is associated with distinct transcriptional changes [123, 168]. In solid tumors, it has been increasingly appreciated that the TAM phenotype in vivo is a continuum between the M1 and M2 dichotomous spectrum [192].

Several studies have identified TNC as an endogenous modulator to promote macrophage and microglial polarization towards M1 pro-inflammatory phenotype, mainly because TNC induces the production of pro-inflammatory cytokines through toll-like receptor 4 [12, 66, 118, 119, 130, 159] and integrins α9β1 [99] and αVβ3 [184], three main receptors to mediate the immunomodulation function of TNC. In an arthritic joint model, TNC-deficient mice displayed decreased sustentation of inflammation and therefore a protection against joint destruction. This finding led to the identification of TNC as an inducer of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α. This study also showed

that the FBG domain of TNC mediated its effect by binding to TLR4 on primary human macrophages [130]. TNC and LPS stimulation in primary murine microglia led to comparable results [66]. In addition to TLR4, binding of TNC to integrins α9β1 and αVβ3 increased the expression of pro-inflammatory cytokines and chemokines, including IL-6, IL-1β, TNF-α, and CCL2 in murine macrophages [99, 184].

When activating TLR4, the FBG domain of TNC does not utilize the classical LPS co-receptors myeloid differentiation protein-2 (MD-2) and CD14 [130]. Comparative analysis of the signaling induced by TNC and LPS has shown that both induced overlapping pathways, such as c-Jun N-terminal kinase (JNK), NF-kB, and MAPK p38; however, each mediated different downstream effects in macrophages. In these experiments, exogenous TLR4 activation in macrophages through LPS resulted in an aggressive anti-microbial phenotype, characterized by the upregulation of various MMPs. Endogenous activation of TLR4 through the FBG domain of TNC displayed matrix-synthesizing activities and phosphorylation of collagen [160].

Further supporting a role of TNC in M1 polarization of macrophage, Piccinini et al. demonstrated that the expression of pro-inflammatory cytokine was impaired in TNC-deficient macrophages due to post-transcriptional modifications mediated by microRNA-155 [160]. Recent studies have reported that TNC suppresses M2 polarization markers and shift macrophages towards M1 polarization by inhibiting interferon regulatory factor 4 (IRF4), a key transcriptional factor that controls M2-polarization in macrophages [107]. The stimulus-based induction of pro-inflammatory cytokines is only part of TNC's role in immunomodulation. In the bone marrow microenvironment, TNC played an essential role during hematopoietic regeneration [142]. Characterization of TNC-deficient mice revealed an important regulatory function of cell-intrinsic expression of TNC.

Besides promoting an M1-like, pro-inflammatory phenotype of macrophages, others have reported the capacity of TNC to mediate M2 polarization of macrophages. Activating transcription factor 3 (ATF3) was induced in various lesions and has been characterized as a regulator of macrophage polarization [181]. Upon upregulation, ATF3 suppressed M1 activity and promoted M2 marker expression and macrophage migration. Overexpression of ATF3 induced the upregulation of TNC through the Wnt/β-catenin signaling pathway, which subsequently acted as an effector in support of the M2 phenotype. Taken together, these studies again indicate multifunction of TNC and suggest that TNC modulates macrophage polarization in a stimulus-based manner.

Phagocytosis While there are many reports investigating TNC's function in macrophages, the impact of TNC on brain residential microglia is less explored. Tenascin-C has been previously suggested to affect microglia in Alzheimer's disease [205] and globoid cell leukodystrophy (GLD) [40]. Consistent with the pro-inflammatory role of TNC in macrophages, Xie et al. reported that TNC promoted the pro-inflammatory response of the AD mouse brains, whereas TNC deficiency enhanced the anti-inflammatory activation [205]. Works from Claycomb et al. found that TNC promoted microglia to shift towards an M1-like phenotype [40].

A recent publication by Ma et al. revealed a novel relationship between TNC and phagocytosis in brain tumors [119]. Loss of the "don't eat me" signal CD47 in GBM xenografts displayed smaller tumors, higher infiltration of TAMs, and increased phagocytosis, accompanied by a dramatic increase in tumor-derived TNC. Furthermore, a high TNC level was associated with an invasive phenotype of tumors with an irregular tumor border, whereas low TNC level was associated with noninvasive, ball-like tumors with a clear-demarcated border. The irregular morphology of tumor border in CD47 KO gliomas could be reminiscent of microglia/macrophages attacking tumor cells at the border. Further analysis confirmed that in murine and human CD47-knockout (KO) glioma cells, TNC was significantly upregulated. Exogenous TNC upregulated TNF-α, IL-6, and IL-1β in human

macrophage cells. Knocking down TNC in CD47-KO glioma cells rescued the impact of CD47 deficiency, with overall larger tumors, less infiltration of TAMs, decreased phagocytosis in vitro, and suppressed inflammation in vivo. These findings are consistent with a previous report from the same group showing that TNC knockdown altered microglial morphology and increased the growth of GBM xenografts derived from GBM neurosphere cultures [204]. Supporting the role of TNC in phagocytosis, Haage et al. recently reported that TNC promoted the phagocytosis capacity of microglia during early postnatal development [66].

Collectively, these studies suggest that TNC may have the capacity to target tumor cells by increasing pro-inflammatory cytokines and promoting the phagocytosis capacity of microglia/macrophage. This concept is consistent with the pro-inflammatory function of TNC in other diseases but stands in contrast to the overall pro-tumorigenic function of TNC. One explanation for the discrepancy between the pro-inflammatory/anti-tumor function of TNC and their high expression in high-grade gliomas is that in these brain tumor animal models, TNC was only modulated in tumor cells but not in host stromal cells. Another explanation is the functional dichotomy of TNC during brain tumor development: a pro-inflammatory, anti-tumor effect at tumor initiation and a tissue remodeling, pro-tumor effect during tumor progression. TNC at the initial phase of tumorigenesis may function as a host-defense mechanism by promoting the expression of pro-inflammatory factors and phagocytosis. Yet, TNC seems to form a self-feedback loop with these inflammatory factors to promote its persistent expression, and some of these TNC-induced inflammatory factors have been heavily linked to tumor progression, such as IL-1 β and IL-6 ([13]; [111]). This explanation is supported by the notion that cancer is heavily associated with chronic inflammation, hence a "wound that never heals" [22]. Thus, elevated TNC level in high-grade gliomas is a reflection of the extent of tissue damage and disease severity.

Other Functions TNC expression in cardiomyopathic diseases showed that local TNC propagated fibrosis through increasing extra-vassal infiltration of inflammatory monocytes. However, reciprocal bone marrow (BM) transplantation of wild-type and TNC-deficient mice resulted in alleviated disease progression in mice with wild-type BM [3, 189]. TNC has also been shown to progress atherosclerotic lesions through activating macrophages. In macrophage-enriched atherosclerotic plaques, TNC was significantly highly expressed and correlated with the degree of inflammation [199]. Accumulating macrophages could progress the formation of plaques in a TNC-dependent manner, through differentiating to foam cells by TLR4 and LDL-uptake receptor CD36 [118].

Similar to TNC, other ECM components, such as HA and versican, have also been known to modulate macrophage activity. Tumor-derived HA modulated macrophage activities and induced the M2 polarization by upregulating immunosuppressive factors, such as IL10, TGF-β, and STAT3 signaling ([106]; [110]; [221]). HA is also critical in the interaction between cancer stem cells and TAMs in breast cancer models. Inhibition of HA production could reduce the pro-tumorigenic phenotype of the tumor microenvironment and reduce bone metastasis [149]. Versican, another endogenous ligand of toll-like receptors, has been shown to be elevated in glioma. Glioma-derived versican could increase the expression of membrane-type MMP14 in murine microglia and modulate destructive matrix properties [84].

9.7.2 Tenascin-C Function in T Cells

Besides TAMs, the immune landscape of the glioma microenvironment constitutes of various cells of the adaptive immune system, including T helper (Th), T cytotoxic (Tc), and regulatory T cells (Treg) [60]. Glioma cells exhibit a multitude of mechanisms to suppress the activity of cytotoxic T cells through expressing immune-

suppressive cytokines, e.g., IL-10 and TGF-β; increasing infiltration of suppressive Tregs, tumor-associated myeloid cells (MDSCs), and M2-like polarized TAMs; and upregulating the expression of immune checkpoints in neoplastic and non-neoplastic cells [158]. T cells distribute in the peritumoral brain zone (PBZ) and in the tumor core of GBM differently. The accumulation of cytotoxic T cells in the PBZ and Tregs in the tumor core provides yet another mechanism for immune evasion [194].

The GBM-associated oncogenic ECM plays an important part in modulating T cell functions. In human GBM specimens, TNC is widely deposited in the vicinity of T cells. In contrast to the overall immune-stimulatory function of TNC on innate immune cells, there have been several reports showing that the effect of TNC on T cells is immune suppressive [77, 81, 161, 169]; however, very little is known about the mechanisms in tumor biology. Adhesion and interaction of lymphocytes with their surrounding ECM through integrins play a crucial role in T cell activity and the ability to migrate [74, 75, 183]. Soluble TNC interfered with integrins α4β1 and α5β1 and inhibited the adhesion of T lymphocytes to fibronectin through its FNIII 1–5 [75]. In transwell migration assays, suppression of TNC expression could reinstate permissive migration of T cells, suggesting that TNC could actively arrest T cells [85]. Arrested T cells were associated with an abnormal morphology, characterized by short podia-like protrusions. Restoring the ERK signaling that was disrupted by TNC rescued migration and morphology of T cells. In addition, TNC bound and stabilized the growth factor TGF-β to provide an indirect immunosuppressive mechanism to inhibit T cells [44]. TGF-β can sometimes elicit stimulatory effects in human T cells by regulating their proliferation and differentiation, depending on the T cell differentiation status and the stimulation conditions [97, 158]. Thus, TNC's binding to TGF-β negatively impacted T cell functions as TGF-β was more than just an immunosuppressive cytokine but had the potential to contribute to anti-tumor immunity.

Consistent with these results, TNC secreted by cancer stem-like cells in prostate cancer inhibited T cell activation, proliferation, and cytokine production by interacting with integrin α5β1 [89]. Analysis of TNC expression in tumor and non-tumor tissues of patients with non-small cell lung cancer (NSCLC) revealed eightfold elevation of the expression of the large isoform of TNC in tumor tissues, which inhibited the proliferation and cytokine production of tumor-infiltrating lymphocytes isolated from the lung cancer specimens [154].

Exosomes are small extracellular vesicles (EVs) secreted by tumor cells, which meditate cell-to-cell interaction by transporting proteins, mRNAs, and microRNAs [10]. Mirzaei et al. reported that TNC was associated with exosomes to influence T cell functions [136]. GSCs have been shown to export EVs filled with TNC and suppress T cell activity in co-cultures. Suppressed murine and human T cells by TNC could no longer inhibit the neurosphere formation of GSCs. The immunosuppressive effect of TNC was mediated through integrins α5β1 and αvβ6, which subsequently resulted in impaired mTOR signaling [136].

9.7.3 Tenascin-C Function in Neutrophil Granulocytes

Several reports suggest that TNC might modulate the innate immune system beyond microglia and macrophages in the tumor microenvironment. Neutrophil granulocytes are acute inflammatory innate immune cells and the first line of defense against infection [122]. Based on neutrophil activity and maturation, they can either promote or suppress glioma progression [187]. Infiltration of neutrophils in GBM has been associated with promotion of GSC proliferation, a negative prognostic value and anti-angiogenic therapy resistance [53, 116]. TNC-deficient mice in a liver ischemia/reperfusion injury (IRI) and liver fibrosis model showed decreased recruitment of neutrophil granulocytes to the injury site [49, 103]. Furthermore, exogenous stimulation of neutro-

phils with TNC increased the expression of matrix modulatory enzymes, such as MMP9, via TLR4 [112].

9.7.4 Tenascin-C Function in Dendritic Cells

Dendritic cells (DCs) secret various pro-inflammatory cytokines and take the role as "professional" antigen-presenting cells (APCs) and therefore play a crucial role in mediating the cross-talk between the innate and adaptive immune system [153]. In glioma, their potent modulatory function of adaptive immune cells has led to various vaccine-based approaches to treat GBM patients in phase III clinical trials [8, 117]. Similar to microglia and macrophages, TNC-deficient mice displayed DCs with decreased production of inflammatory cytokines, such TNFα and IL6, upon LPS stimulation [170]. Furthermore, they exhibited specific defects in Th17 polarization of T cells and decreased IL17 expression in vitro and in vivo. DCs stimulated with TNC produced increased pro-inflammatory cytokines and consequently promoted Th17 differentiation via TLR4 signaling [120]. Overall, TNC expression directly leads to an increased inflammatory reaction of yet another type of innate immune cells (DCs) and indirectly modulates the adaptive immune system (Th17).

9.7.5 Tenascin-C Function in Natural Killer Cells

Natural killer (NK) cells are lymphocytes of the innate immune system to modulate immune response by secreting cytokines and chemokines, as well as mediating antigen-independent cell cytotoxicity [30]. Similar to T cells, their activity in the tumor microenvironment is suppressed by Tregs, MDSCs, and TAMs [121]; therefore, reinstating their anti-tumor cytotoxicity has been a prominent approach in GBM immunotherapy [63]. Human natural killer-1/CD57 (HNK-1)-positive NK cells have been previously characterized with high cytotoxic potential [100]. $CD57^{+}$-NK cells correlate with increased survival in patients with leukemia, lymphoma, carcinoma, and melanoma [5, 9, 146]. Interestingly, HNK-1 expression on neural stem cells was linked to the largest isoform of TNC [206]. This might suggest a possible interaction between TNC expression in the tumor microenvironment and NK cell cytotoxicity.

9.8 Translation into the Clinic

9.8.1 Tenascin-C in Adjuvant Anti-cancer Therapy

High tissue level of TNC correlates with poor patient survival and disease progression in various solid malignancies including GBM, making it a good candidate as a tumor-specific antigen and a prognosis indicator for anti-cancer therapy [41, 61, 113, 114, 148, 162, 210, 211]. A substantial body of work is utilizing this high-expression property of TNC in tumors to design TNC-specific antibodies and aptamers (oligonucleotides, e.g., DNA or RNA, that specifically bind to TNC) to deliver anti-tumor drugs directly to the tumor microenvironment [190]. Specifically, antibodies targeting TNC have been used for selective transport of anti-cancer reagents to target brain tumor cells, including immunostimulatory chemokines/cytokines, chemotherapeutics, and radiotherapeutic agents [6, 26, 65, 164, 165, 218]. For example, F16, an antibody that recognizes the FNIII domain of TNC, has been shown to concentrate in GBM [157] and numerous other cancer types [14, 55, 156, 178, 180]. This approach has also been used for PET/CT imaging of brain tumors [1, 38, 80, 90].

9.8.2 Tenascin-C as a Biomarker

The disease-associated expression pattern of TNC in adult tissues provides a great potential for TNC as a biomarker in diagnostic applications. TNC also circulates in bodily fluids, includ-

ing serum [177] and breast milk [54]. Measurement of TNC in serum revealed an increase of TNC in patients with glioma and several inflammatory diseases, in which TNC correlated with c-reactive protein (CRP) expression [162, 166, 177, 219]. Mock et al. [138] employed printed peptide arrays to detect serum antibodies and found that elevated level of antibodies against a specific peptide located in the EGF-like domain of TNC was associated with prolonged survival of GBM patients. Higher concentrations of TNC in synovial fluid were linked to the progression of osteoarthritis [71] and rheumatic arthritis [72]. In patients with hypertrophic cardiomyopathy, serum TNC was also a prognostic biomarker for heart failure [108]. Taken together, TNC is not only considered as a potential biomarker in tissues but also in the noninvasive approachable circulatory system.

9.8.3 Inhibiting Tenascin-C

TNC mediates pleiotropic effects in the tumor microenvironment [127, 132], which makes it an interesting target for therapy. Small interfering RNA (siRNA) designed to inhibit TNC expression in human glioma was directly injected into a small group of 11 patients with low- grade and high-grade gliomas after resection. Treated patients showed slower relapse, longer survival, enhanced growth inhibition, and even almost recession in some cases with no noticeable neurological deficits [225]. A follow-up study with a larger patient cohort of 46 displayed a significant improvement in overall survival when compared with 48 patients with only brachytherapy: a prolonged survival of 4.8 weeks in grade II, 13.2 weeks in grade III, and 13.9 weeks in grade IV gliomas when treated with TNC siRNA. Furthermore, MRI and CT studies showed delayed tumor growth and decreased tumor recurrence without the loss of quality of life [167, 203]. Additionally, a multi-peptide vaccine IMA950 that targets TNC showed promising results in the overall survival and safety of GBM patients [134].

9.9 Conclusion and Future Directions

There is no doubt that TNC plays an important role in physiology and pathology due to its tightly controlled expression pattern. TNC is involved in stem cell maintenance during development, and high expression of TNC is associated with infection, inflammation, cancer progression, and metastasis. TNC interacts with a myriad of partners to influence cell adhesion, migration, proliferation, and angiogenesis and, most importantly, serves as a member of the DAMP signals to modulate immune responses. Literature shows that TNC displays a considerate degree of functional dichotomies in many aspects of TNC-mediated cellular functions, including cell adhesion, proliferation, and immunomodulation. Challenges will include elucidating the context-dependent function of TNC isoforms. Although alternative splicing and post-translational modifications of TNC have been identified, their function is poorly understood, especially in immune modulation. Although TNC is heavily involved in infection and inflammation, its immunomodulation role in cancer is less appreciated. The details of how TNC modulates signaling pathways to regulate immune responses in a wide range of cell types remain largely unknown. For example, it is still unknown how TNC exactly interacts with TLR4. Immunocompetent animal models for solid tumors with tightly controlled, tissue-specific expression of TNC will help to dissect the function of TNC in vivo, a better understanding of which will ultimately lead to effective combinatory therapies again tumor growth and metastasis.

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