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Alexander Birbrair *Editor*

Tumor Microenvironment

Extracellular Matrix Components – Part A

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Alexander Birbrair
Editor

Tumor Microenvironment

Extracellular Matrix Components – Part A

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Editor
Alexander Birbrair
Department of Radiology
Columbia University Medical Center
New York, NY, USA

Department of Pathology
Federal University of Minas Gerais
Belo Horizonte, MG, Brazil

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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 A* 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 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 tumor microenvironment in several tissues using state-of-the-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. Eight chapters written by experts in the field summarize the present knowledge about distinct extracellular matrix constituents during tumor development.

Maria Angelica Miglino and colleagues from the University of Sao Paulo wrote an introductory chapter on the role of different extracellular matrix components in the tumor microenvironment. Anthony J. Hayes and James Melrose from The University of Sydney discuss the role of keratan sulfate within the tumor. Laura Alaniz and colleagues from the Universidad Nacional del Noroeste de Buenos Aires compile our understanding of hyaluronan in the tumor microenvironment. Peter Qiao and Zheng-Rong Lu from Case Western Reserve University update us with what we know about fibronectin in the tumor microenvironment. Georgina Gonzalez-Avila and colleagues from Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" summarize current knowledge on matrix metalloproteinases role in

tumor microenvironment. Mary C. Farach-Carson and colleagues from The University of Texas Health Science Center at Houston describe the influence of perlecan and its modifiers in the tumor microenvironment. Evgenia Karousou and colleagues from the University of Insubria address the importance of heparan sulfate in the tumor microenvironment. Finally, Brad Walsh and colleagues from Glytherix Ltd. give an overview of the role of glypican-1 in the tumor microenvironment.

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
Belo Horizonte, MG, Brazil

Alexander Birbrair

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Contributors

Laura Alaniz Laboratorio de Microambiente Tumoral, Centro de Investigaciones Básicas y Aplicadas (CIBA), Universidad Nacional de la Pcia. de Bs. As. Centro de Investigaciones y Transferencia del Noroeste de la Pcia. de Bs. As. (CIT NOBA, UNNOBA-CONICET), Junín, Buenos Aires, Argentina

Barbara Bartolini Department of Medicine and Surgery, University of Insubria, Varese, Italy

Alexander Birbrair Department of Radiology, Columbia University Medical Center, New York, NY, USA

Department of Pathology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Jessica Borghesi Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

Douglas H. Campbell GlyTherix Ltd, Sydney, NSW, Australia

Ilaria Caon Department of Medicine and Surgery, University of Insubria, Varese, Italy

Elena Caravà Department of Medicine and Surgery, University of Insubria, Varese, Italy

Lissette A. Cruz Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston, Houston, TX, USA

Gustavo de Sá Schiavo Matias Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

Mary C. Farach-Carson Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston, Houston, TX, USA

Paula Fratini Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

A. Armando García-Hernández Laboratorio de Oncología Biomédica, Instituto Nacional de Enfermedades Respiratorias “Ismael Cosío Villegas”, Mexico City, Mexico

Georgina Gonzalez-Avila Laboratorio de Oncología Biomédica, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

Anthony J. Hayes Bioimaging Research Hub, Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, UK

Evgenia Karousou Department of Medicine and Surgery, University of Insubria, Varese, Italy

Maria E. Lund GlyTherix Ltd, Sydney, NSW, Australia

Zheng-Rong Lu Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA

James Melrose Graduate School of Biomedical Engineering, University of New South Wales, Sydney, NSW, Australia

Raymond Purves Laboratory, Institute of Bone and Joint Research, Kolling Institute, Northern Sydney Local Health District, Royal North Shore Hospital, St. Leonards, NSW, Australia

Sydney Medical School, Northern, The University of Sydney, Faculty of Medicine and Health at Royal North Shore Hospital, St. Leonards, NSW, Australia

Maria Angelica Miglino Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

Paola Moretto Department of Medicine and Surgery, University of Insubria, Varese, Italy

Arianna Parnigoni Department of Medicine and Surgery, University of Insubria, Varese, Italy

Alberto Passi Department of Medicine and Surgery, University of Insubria, Varese, Italy

Concepta Margaret Mc Manus Pimentel Department of Physiological Science, Institute of Biological Science, University of Brasilia, Brasilia, Brazil

Pedro Henrique Dias Moura Prazeres Department of Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Peter Qiao Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA

Ana Carolina Silveira Rabelo Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

Carlos Ramos Laboratorio de Biología Celular, Departamento de Fibrosis Pulmonar, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

Nathia Nathaly Rigoglio Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

Ina Sevic Laboratorio de Microambiente Tumoral, Centro de Investigaciones Básicas y Aplicadas (CIBA), Universidad Nacional de la Pcia. de Bs. As. Centro de Investigaciones y Transferencia del Noroeste de la Pcia. de Bs. As. (CIT NOBA, UNNOBA-CONICET), Junín, Buenos Aires, Argentina

Bettina Sommer Departamento de Investigación en Hiperreactividad Bronquial, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

Fiorella Mercedes Spinelli Laboratorio de Microambiente Tumoral, Centro de Investigaciones Básicas y Aplicadas (CIBA), Universidad Nacional de la Pcia. de Bs. As. Centro de Investigaciones y Transferencia del Noroeste de la Pcia. de Bs. As. (CIT NOBA, UNNOBA-CONICET), Junín, Buenos Aires, Argentina

Tristen V. Tellman Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston, Houston, TX, USA

Davide Vigetti Department of Medicine and Surgery, University of Insubria, Varese, Italy

Manuela Viola Department of Medicine and Surgery, University of Insubria, Varese, Italy

Daiana Lujan Vitale Laboratorio de Microambiente Tumoral, Centro de Investigaciones Básicas y Aplicadas (CIBA), Universidad Nacional de la Pcia. de Bs. As. Centro de Investigaciones y Transferencia del Noroeste de la Pcia. de Bs. As. (CIT NOBA, UNNOBA-CONICET), Junín, Buenos Aires, Argentina

Bradley J. Walsh GlyTherix Ltd, Sydney, NSW, Australia

The Tumor Microenvironment: Focus on Extracellular Matrix

1

Nathia Nathaly Rigoglio, Ana Carolina Silveira Rabelo, Jessica Borghesi, Gustavo de Sá Schiavo Matias, Paula Fratini, Pedro Henrique Dias Moura Prazeres, Concepta Margaret Mc Manus Pimentel, Alexander Birbrair, and Maria Angelica Miglino

Abstract

The extracellular matrix (ECM) regulates the development and maintains tissue homeostasis. The ECM is composed of a complex network of molecules presenting distinct biochemical properties to regulate cell growth, survival, motility, and differentiation. Among their components, proteoglycans (PGs) are considered one of the main components of ECM. Its composition, biomechanics, and anisotropy are exquisitely tuned to reflect the physiological state of the tissue. The loss of ECM's homeostasis is seen as one of the hallmarks of cancer and, typically, defines transitional events in tumor progression and metastasis. In this chapter, we discuss the types of proteoglycans and their roles in cancer. It has been observed that the amount of some ECM components is increased, while others are decreased, depending on the type of tumor. However, both conditions corroborate with tumor progression and malignancy. Therefore, ECM components have an increasingly important role in carcinogenesis and this leads us to believe that their understanding may be a key in the discovery of new antitumor therapies. In this book, the main ECM components will be discussed in more detail in each chapter.

Keywords

Extracellular matrix · Cancer · Components · Proteoglycan · Extracellular matrix proteases · Tumor microenvironment · Metastasis · Growth · Progression · Angiogenesis

N. N. Rigoglio · A. C. S. Rabelo · J. Borghesi · G. de Sá Schiavo Matias · P. Fratini · M. A. Miglino (✉)
Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil
e-mail: miglino@usp.br

P. H. D. M. Prazeres
Department of Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

C. M. M. M. Pimentel
Department of Physiological Science, Institute of Biological Science, University of Brasilia, Brasilia, Brazil

A. Birbrair
Department of Radiology, Columbia University Medical Center, New York, NY, USA

Department of Pathology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

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1.1 Introduction

The extracellular matrix (ECM) is a well-organized network formed by a mixture of non-cellular components present in all organs and tissues. It is able to regulate many cellular processes besides providing biological scaffolds [73, 117, 394]. Proteoglycans (PGs) are the major components of ECM being also the most important among the structural proprieties from tissues and cells. PGs constitute a large family, being subdivided into several subclasses, as outlined in Fig. 1.1. Through their glycosaminoglycans (GAGs) and core proteins, PGs interact with numerous growth factors, cytokines and chemokines, cell surface receptors, and ECM molecules [445]. Several cellular functions, such as signaling, proliferation, migration, differentiation, apoptosis, and cell adhesion, are regulated by PGs, which are also important in the organization of ECMs, due to their ability to interact with other ECM molecules and cells. In homeostasis and during tumorigenesis, PGs are essential as their biosynthesis is markedly modified during ECM remodeling [168, 169, 393, 395, 396] (Fig. 1.2).

1.2 Extracellular Proteoglycans

1.2.1 Hyalectans

It is a subfamily of proteoglycans represented by aggrecan, versican, neurocan, and brevican that share common structural features. The N-terminal domain of these PGs contains Ig-like repeat followed by link-protein-like modules (PG tandem repeats) that through disulfide-bonded Cys form globular domains (G). All hyalectans have a G1 domain; however, an additional G2 is only present at aggrecan. A variable number of potential GAG attachment sites are present in their central domain, whereas the C-terminal domain contains the G3 globular domain. Brevican contains only one epidermal growth factor (EGF)-like repeat, the G3 contains two EGF-like repeats, a C-type lectin domain, and a complement regulatory protein domain. The N-terminal domain enables the connection of hyalectans to hyaluronan HA, whereas the C-terminal domain binds to lectins [167].

1.2.1.1 Aggrecan

Aggrecan is a chondroitin sulfated proteoglycan (CSPG), a critical structural component of cartilage; however, it is also expressed in the intervertebral disk and brain. A unique feature of this protein family is that it forms a large aggregated structure by binding to hyaluronan (HA), a high-molecular-weight carbohydrate polymer present in the ECM. Aggrecan has three globular domains (G1, G2, and G3) and three extended domains [interglobular domain (IGD), keratan sulfate (KS), and chondroitin sulfate (CS)]. N-terminal G1 domain contains a signal peptide; an IGD does the connection between G1 and G2, and there is a large sequence between G2 and G3 domains, which is modified by KS and CS side chains [190].

Maintenance of the aggregable aggrecan content in cartilage is critical to the function of the tissue. The loss of aggrecan, which prevents the diffusion of high-molecular-weight molecules into the cartilage matrix, may allow increased diffusion of small molecules, such as cytokines and proteases, from the surrounding epithelia or fluids into the cartilage leading to its further erosion [359]. Studies have reported that aggrecan is depleted in laryngeal cancer [360, 378] and prostate cancer [381].

1.2.1.2 Versican

Versican, a large chondroitin sulfate proteoglycan (CSPG), is expressed in various tissues with versatile functions. Chondroitin sulfates (CSs) are a group of sulfated GAGs composed of two alternating sugars [396]. Its central domain consists of two large subdomains (GAGα and GAGβ), which can be alternatively spliced in four different variants and differentially expressed around the tissues [169, 324, 390, 396]. Mutation in the versican gene can result in some anomalies, such as autosomal dominant eye disorders, denominated Wagner syndrome, and erosive vitreoretinopathy [266]. Recently, a novel variant

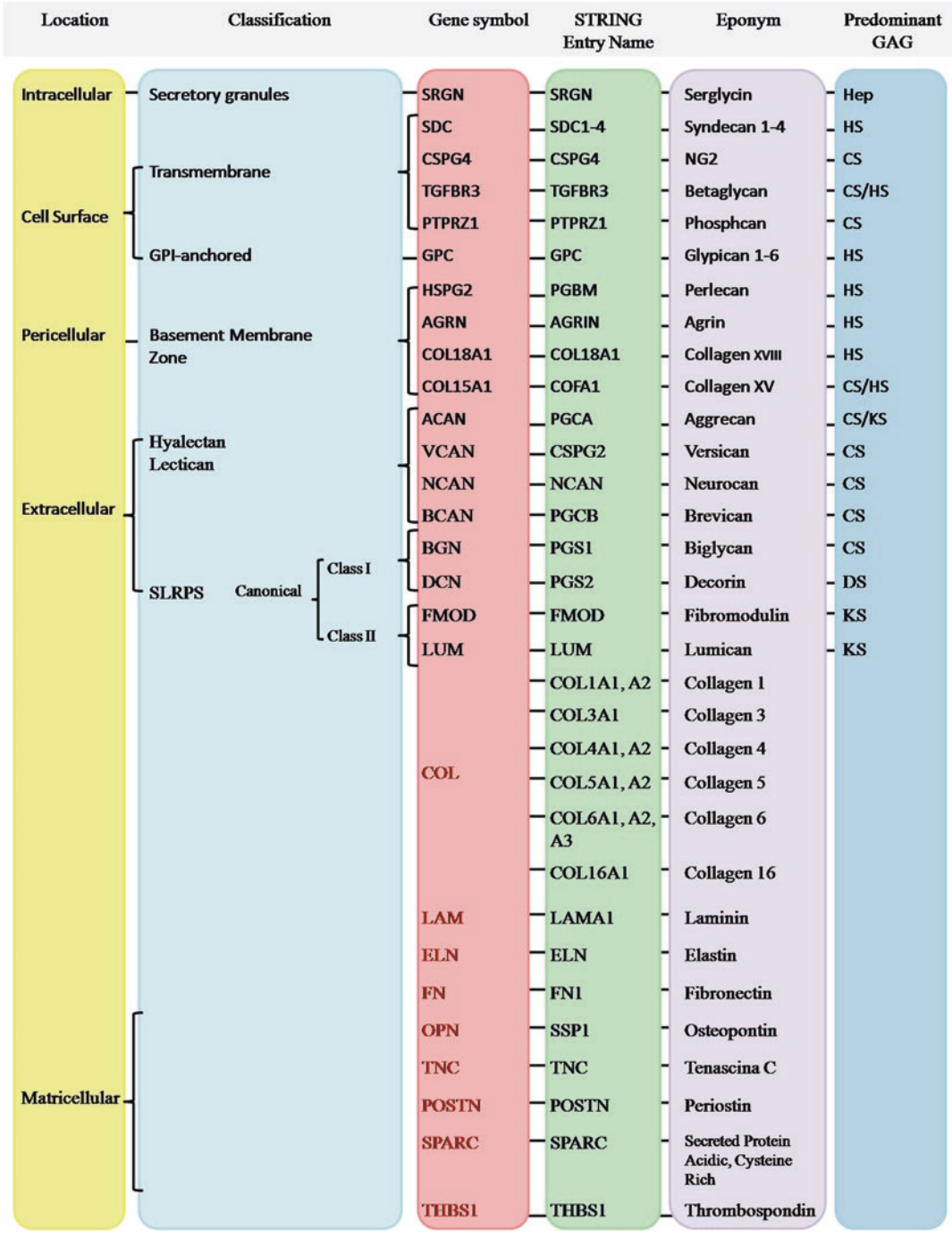

Fig. 1.1 Proteoglycans are divided into: intracellular, cell surface, pericellular basement and extracellular, as well as collagen, laminin, elastin, fibronectin. In addition to matricellular proteins: osteopontin, tenascin C, periostin, secreted protein acidic and cysteine rich, thrombospondin

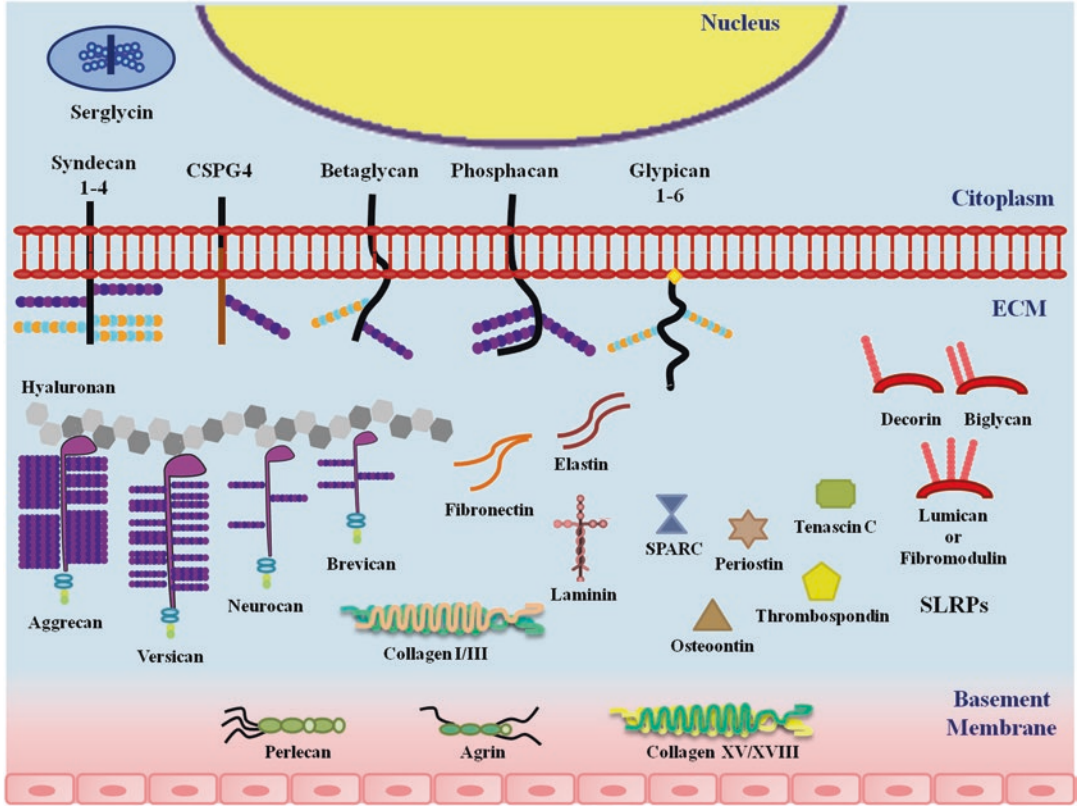

Fig. 1.2 Schematic representation of proteoglycans classification as extracellular, pericellular, cell surface and intracellular (proteins localization, homology, and genomic levels). The serglycin, an intracellular proteoglycan, is found in secretory vesicles of hematopoietic and endothelial cells. Cell surface proteoglycan comprises four syndecans, CSPG4/NG2, betaglycan, phosphacan, and six glypicans. There are three groups of extracellular proteoglycans: (1) hyalectans (aggregan, versican, neurocan, and brevican), which are associated with hyaluronan in the ECM; (2) SRLPs (decorin, biglycan, lumican, and fibromodulin); (3) basement membrane proteoglycans (perlecan, agrin, collagen XV and XVIII). In the extracellular matrix, proteins such as collagen, elastin, fibronectin, and laminin are also found. The matricellular proteins (osteopontin, periostin, SPARC, tenascin C, and thrombospondin) are non-structural proteins present in the ECM

that contains part of the GAGβ domain and is partially glycanated has been detected in human breast cancer tissues [200].

Versican regulates cell adhesion, migration, and inflammation [431, 432]. The activation of fibroblasts in the tumoral process causes the accumulation of versican in the stroma. Some tumor cells also express versican, which leads to augmented tumorigenesis [396]. Increased metastasis formation may occur due to the interaction of versican with TLR2 present in macrophages derived from bone marrow, which induce the secretion of tumor necrosis factor α (TNF-α) [196]. Activation of epidermal growth factor receptor (EGFR) signaling by versican EGF-like repeats promotes the growth, migration, invasion, and resistance to therapies [390, 396]. Interestingly, overexpression of versican can enhance self-renewal and stem cell properties through EGFR signaling [96].

CSPGs play an important role in breast cancer progression [396]. The accumulation of versican within the ECM of the peritumoral stroma is observed in human breast cancer, and correlates with poor relapse-free survival in patients [200, 333]. In vitro experiments show that secreted factors from cancer cells induce the expression and secretion of versican by fibroblasts [333].

Therefore, versican is considered an important modulator of cellular behavior in breast cancer and has been shown to promote cancer cell survival, tumor growth, and bone metastasis when overexpressed [452]. Versican also promotes the formation of mammospheres and cancer cell colonies in vitro and has been shown to support breast cancer tumorigenesis in vivo by increasing the expression of stem cell markers such as aldehyde dehydrogenase 1 (ALDH1), CD44, and integrin-β1 [96].

1.2.1.3 Neurocan and Brevican

Neurocan can carry up to seven CS chains and is expressed in the brain. In addition to inhibiting neurite outgrowth in vitro, its expression is increased at the site of mechanical and ischemic injury [85, 169, 229]. The role of neurocan in cancer is not yet fully understood; however, it has already been demonstrated that in neuroblastoma cells its overexpression in vitro and in vivo potentiates tumor cell growth [380]. Brevican is also expressed in the brain, specifically in the outer surface of neurons and is enriched at perisynaptic sites. Brevican was correlated with nervous tissue injury and repair, Alzheimer's disease, and also promotes growth and progression of gliomas [100, 169, 235].

1.2.2 Small Leucine-Rich Proteoglycans (SLRPs)

This is the largest family of PGs containing 18 members grouped into five classes and ubiquitously expressed in most ECMs. The most studied subtypes involved in tumorigenesis are decorin, biglycan, fibromodulin, and lumican [169]. SLRPs share many biological functions, such as interaction with various collagen, binding to tyrosine kinase receptors and innate immune receptors [65, 98, 155, 169, 263, 361].

1.2.2.1 Decorin

Decorin is covalently linked with a linear GAG chain, which can be CS or DS. This protein receives this name due to its propensity to decorate collagen fibrils. Decorin core protein binds non-covalently to an intraperiod site of 67 nm (D period) on the surface of collagen fibrils. In addition, the alignment of collagen fibrils has the participation of the decorin's GAG chain that also has a crucial regulatory effect on collagen fibrillogenesis. Decorin may be considered important for the mechanical properties of various connective tissues, as well as for the maintenance of corneal transparency [285, 416]. Mutations in the decorin gene were associated with congenital stromal corneal dystrophy syndrome [43, 198].

Besides acting as a tumor suppressor molecule, decorin is highly present in the tumor stroma. TGF-β is one of the growth factors with which decorin interacts, causing inhibition of cell proliferation [449]. Decorin also inhibits EGFR by partially overlapping with an EGF-binding epitope, thereby blocking the growth of a variety of cancer cells via expression of $p21^{WAF1}$ [88, 170, 265]. Moreover, this binding promotes sustained downregulation of EGFR in vivo by controlling tumor cell growth [81, 471]. Decorin stimulates the expression of anti-angiogenic molecules, such as thrombospondin 1 (TSP-1) and tissue inhibitor of metalloproteinases-3 (TIMP-3), and inhibits pro-angiogenic factors like hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor A (VEGFA) [48, 131, 274, 275]. Decorin also suppresses insulin-like growth factor-I receptor (IGF-IR) [263, 264] and vascular endothelial growth factor receptor 2 (VEGFR2) [274]. This type of binding evokes autophagy in endothelial cells through partial agonist activity of VEGFR2 and induction of Peg3 [49]. In summary, decorin has multiple inhibitory roles in the growth of cancer cells, either suppressing growth factor receptors and angiogenesis [175] or inducing prolonged stromal cell autophagy [276].

The characteristics of decorin made it interesting as a breast tumor and metastasis suppressor [274]. There is a significant expression of decorin in the healthy mammary gland, while its expression is reduced in breast cancer [107, 221, 292]. In a model of lung metastasis of breast cancer, the overexpression or systemic administration of recombinant decorin led to a significant reduction of expression and activation of ErbB2, as

well as inhibiting tumor growth and metastasis [10, 131, 331]. Tumor progression can be prevented when there is a reduction in the availability of activated TGFβ1, since decorin affects TGFβ signaling [367]. It has also been shown that in breast cancer cells, decorin can induce a state of mitofagia in which the selective degradation of mitochondria occurs through autophagy. This process occurs through decorin-dependent induction of the mitochondrial protein and the putative tumor suppressor mitostatin [276]. A good prognosis was observed in a cohort of 140 breast cancer patients when decorin levels were clinically evaluated, corroborating with its antitumor and anti-metastatic properties [404].

1.2.2.2 Biglycan

Biglycan is highly homologous to decorin, once both bind with high affinity to collagen fibril, its amino-terminal domain presents two GAG attachment positions. It is highly present in the dermis and other connective tissues, and plays a key role in postnatal growth [446]. Both are able to bind to TGF-β and inhibit its activity in vitro [26, 204]. Some studies have demonstrated significantly higher expression of biglycan in tumor tissues, including endometrial, pancreatic and colon tumor as well as esophageal squamous cell carcinoma [158]. The upregulation of biglycan in prostate cancer was associated with a poor prognosis [174].

1.2.2.3 Fibromodulin

Although it shares high homology with decorin and biglycan, fibromodulin bears KS-GAG chains linked to asparaginyl residues rather than CS or DS linked to serinyl/threonyl residues. Fibromodulin interacts with type I and II collagens, modulates collagen fibrillogenesis, as well as activating the complement cascade [169, 358]. Deposition of fibromodulin was seen in solid tumors, which promotes the formation of a dense stroma. This change in the density of the stroma causes elevated intersticial fluid pressure (IFP), leading to a decrease in drug delivery and affecting the response to chemotherapy [294]. Furthermore, fibromodulin may also support tumor cell growth by promoting angiogenesis [3, 176].

1.2.2.4 Lumican

Lumican is a leucine-rich proteoglycan that expresses keratan sulfate side chains. It is localized primarily in mesenchymal tissues and tumor stroma [169]. Lumican is highly expressed in breast cancer and melanomas [45, 221, 356]. The expression of lumican in stromal tissues in breast cancer is associated with a high tumor grade, a low estrogen receptor expression level, and young age [221]. The interaction between lumican and β1-containing integrins blocks melanoma cell adhesion [82] and also modulates focal adhesion complexes [45]. It is also expressed in osteosarcoma [281, 283], where it modulates TGF-β2 activity and inhibits membrane type 1-MMP (MT1-MMP) activity [282, 314].

Although lumican expression has been observed within the stroma of breast tumors [220, 221], the analysis of clinical samples from breast cancer patients treated with hormone therapy demonstrated that low lumican expression was associated with poor relapse-free and overall survival [404]. Moreover, in a mouse model of metastatic breast cancer, ectopic lumican expression impaired both primary tumor growth and lung metastasis [353]. These findings may suggest that lumican acts as a tumor suppressor in breast cancer; however, more cellular and molecular details are necessary to determine its role in this context.

1.3 Pericellular-Basement Proteoglycan

1.3.1 Perlecan

Perlecan, also known as heparan sulfate proteoglycan 2 (HSPG2), is a modular PG with five domains and is one of the main components of the ECM [132]. Perclan preferably interacts with β1 integrin and α-dystroglycan receptors in which the differential expression controls proliferation and differentiation [132].

It plays important roles in lipid metabolism, inflammation and wound healing, thrombosis and cancer angiogenesis [140]. Numerous studies have shown that perlecan promotes tumor cell

growth, chemoresistance, migration, and invasion mostly regulating heparin-binding growth factors such as FGF-2, VEGF-A, and Hedgehog (Hh) in prostate cancer, oral squamous cell carcinoma, melanoma [391], and breast and colon cancers [14]. However, it is differentially expressed during matrix remodeling. The expression of perclan has been demonstrated to be important in cases of melanoma, oral squamous carcinoma, prostate desmoplastic carcinoma, and others [95].

Perlecan is a powerful regulator of angiogenesis exhibiting a dual function. The N-terminal domain has pro-angiogenic properties, allowing the vascularization in the tumor stroma, besides acting as a reservoir of angiogenic growth factors sequestering and protecting them from proteolysis within the ECM. However, it is also able to block endothelial cell migration and angiogenesis, since endorepellin (C-terminal domain of perlecan) has distinct sites for the simultaneous binding to integrin α2β1 and VEGFR2 acting as a dual receptor antagonist inhibiting angiogenesis [391]. Endorepellin appears to play a beneficial role as an angiostatic and anti-tumorigenic agent, and can be used as a therapeutic alternative against cancer [30].

1.3.2 Agrin

Agrin is a glycosylated proteoglycan synthesized by motorneurons and secreted in basal lamina that plays an important key role on neuromuscular junction [305], acting on the formation and stabilization of synapses [52]. It is involved in the formation of the blood–brain barrier and also binds to TGFβ family proteins and beta-amyloids [52]. It has already been described that agrin has an extracellular matrix sensor that stabilizes focal adhesions and promotes hepatocellular carcinoma HCC [59].

Agrin is regulated in a variety of diseases [52]. It has also been described that this protein is deposited on the walls of hepatocellular carcinoma blood vessels and contributes to activate the hepatic stellate cell, suggesting its role to promote the process of hepatocarcinogenesis [237]. Although it is known that agrin is produced homogenously in all cancer cells or specifically in a group of cancer cells, it is not yet known if it acts in an autonomous or non-autonomous manner [444].

1.4 Cell Surface Proteoglycans

There are two major classes of cell surface proteoglycans: syndecans and glypicans. The glypicans are attached to the cell membrane through a phospholipid anchor [112] and are therefore not transmembrane. Moreover, a great number of growth factors and morphogens interact with syndecan and glypican's heparan sulfate chains (HS), which are also important co-receptors for them. However, the cell surface proteoglycans do not appear to have a significant role in cell–ECM interactions [50, 111, 112].

1.4.1 Syndecans

Syndecans represent the most expressive family of heparin sulfate proteoglycans (HSPGs) in higher vertebrates. Their functions are correlated with the regulation of many cellular processes: adhesion, migration, and proliferation [79]. Almost all cell types express all four or at least one syndecans, with the exception of erythrocytes. While most sindecans appear to be more widely expressed in the most cell types and tissue, syndecan-3 appears to be more expressed in neural tissue and less in the developing skeletal muscles [442]. Both in normal development and in cancer, morphological transitions and cell differentiation occur, and these involve changes in the pattern of syndecan expression and distribution [8, 135, 406, 456].

Studies have shown that in solid and hematogenous tumors there are changes in syndecan expression, suggesting that syndecan mutations play an important role in tumor progression [439, 457]. The syndecan-1 expression has been related to tumor growth and malignancy in melanoma [326]. It is present in the tumor stroma and breast carcinoma cells, and correlates with poor

prognosis [218]. It has been related to mammary carcinogenesis due to its responsiveness to important regulators of cell growth and proliferation [7]. In other tumors, for example, head and neck and oral squamous cell carcinomas, its loss is related to worse patient outcome [108]. In melanomas, the upregulation of NG2/CSPG4 contributes to an invasive behavior, probably due to its interaction with type VI collagen, which might be a useful tool in prognosis and stratification of patients [57].

Syndecans do not always act independently, since they have been associated with integrins in many cases, but have not yet been shown to be co-receptors with DDR proteins [80]. In different cancers, the region of syndecan-1 ectodomain that interacts with integrins, known as synstatin, can be biologically important, since in human mammary carcinoma cells, synstatin could be a powerful inhibitor [22]. As a result, there is the possibility of new approaches directed to the tumor cell surface receptors which can be applied in all human syndecans. The regulation of β1 integrin may occur, indirectly, through the interaction of syndecan-2's ectodomain with the transmembrane phosphatase CD148 [328]. The association between syndecan-4 and integrins affects cell migration because of its influence in focal adhesions, as well as in the size and number of adhesions [79].

A mechanism that can lead to increased tumor growth and metastasis may occur due to the shedding of syndecan-1 in conjunction with the action of heparanase. The high level of soluble syndecan-1 has a notable impact on tumor growth, as well as in other diseases [123, 223, 373, 384]. Heparanase is an endoglycosidase (endo-β-glucuronidase) which cleaves heparan sulfate (HS) between glucuronic acid and N-sulfoglucosamine residues, thus contributing in degradation and remodeling of the ECM, as well as in cell invasion. The smaller HS fragments resulting from heparanase action have enhanced binding to specific ligands. Many ECM components have Hep-binding domains. The heparanase releases Hep-bound molecular effectors, which bind to growth factors, cytokine, and chemokines. These processes contribute to their dissemination and activity and enhancing cell migration and invasion. In addition, heparanase also releases angiogenic and tumorigenic factors from the ECM [311, 322, 326, 397, 417].

Syndecan-1 (SDC-1) expression is consistently observed in the stroma of human and murine mammary tumors [240, 372], and its high expression predicts poor overall patient survival [216], even in patients treated with systemic chemotherapy [19]. SDC-1 levels predict a reduced pathological response to systemic cyclophosphamide–epirubicinne coadjuvant chemotherapy [136]. The invasiveness of early-stage tumors is promoted by SDC-1, in which overexpression in cancer cells leads to the formation of poorly cohesive and more invasive colonies in 3D cultures [21]. In another study, there was evidence of functional coupling between SDC-1 and αvβ3 integrin, since SDC-1 mediated breast carcinoma cell spreading in an integrin αvβ3-dependent manner [21]. Interestingly, SDC-1 also promoted stem cell properties via regulation of the Wnt and IL-6/STAT-5 pathways [162].

1.4.2 Glypicans

Glypicans are proteoglycans bound to the external surface of the cell membrane. There are six glypican family members in mammals [50, 111, 112]. Its structural features comprise 14 conserved cysteine residues that account for the formation of a compact, globular, N-terminal distal portion of the core proteins. Another characteristic feature of all glypicans is that the GAG chains are located close to the plasma membrane, implying in the interaction of glypican with other cell surface proteins [366].

In breast cancer samples, the expression of glypican 1 (GPC1) is increased [247]. However, downregulation of GPC1 in cancer cells has been related to mitogenic response and contributes to cancer progression [247]. Glypican-3 (GPC3) regulatory role involves a number of cellular functions, besides being considered a tumor suppressor. The hypermethylation of the GPC3 promoter causes silencing of its expression; while

cell growth is inhibited by GPC3 overexpression in breast cancer [443]. Ectopic expression of GPC3 in murine breast cancer has been shown to abrogate primary tumor growth and lung metastasis [309], and also inhibits the PI3K/Akt pathway and strongly induces p38 kinase activity, resulting in increased apoptosis [47], respectively. In vitro, the delay in propagation and reduction of motility occurs due to overexpression of GPC3, which sensitizes the cells to serum starvation induced to apoptosis. Furthermore, GPC3 induces expression of E-cadherin, loss of which is often associated with breast cancer progression [309]. Together, these findings suggest that GPC1 and GPC3 act as a promoter and a suppressor of cancer, respectively [177, 236].

1.4.3 Chondroitin Sulfate Proteoglycan 4 (CSPG4)

Chondroitin sulfate proteoglycan 4 (CSPG4) is a highly glycosylated transmembrane protein, which is also called neural-glia 2 (NG2). It is believed that its expression may play a role in sarcoma initiation since it is a gene expressed by mesenchymal progenitors. The first studies indicated an association of CSPG4 with malignant melanoma. However, other studies have shown its implication in solid tumors and hematological cancers [433]. It presents a restricted/low distribution in normal tissue, but is overexpressed in some types of tumor and at different disease stages, and based on evidence of their multiple roles in both tumor growth and dissemination, presuppose its use as a potential immunotherapeutic target [433].

Chemoresistance and cell survival are activated by NG2/CSPG4-mediated integrin (α3β1) signaling by sustained activation of PI3K/Akt signaling and their downstream targets, especially in human glioblastoma [62, 383, 468]. Studies implicate NG2/CSPG4 as important in facilitating the growth and survival of malignant melanoma due to its wide expression in melanoma cells. The regulation of tyrosine kinase receptor activity is stimulated by activation of the MEK/ERK1,2 pathway due to the action of NG2/CSPG4 that has a cytoplasmic domain. This interaction promotes the intracellular signaling of integrins that play an important role in the activation of survival and growth pathways [451, 460].

NG2/CSPG4 and integrins can be potential cell membrane receptors for collagen VI once it interacts with a variety of cell membranes and ECM proteins. The tumor microenvironment interacts with soft tissue sarcoma cells, thus it is essential that the interaction between CSPG4 and collagen VI occurs [58].

Modulation of cell-ECM and cell-cell interactions may occur because of the localization of NG2/CSPG4 in cancer cells. Cell-ECM interactions can also be regulated indirectly, since NG2/CSPG4 serves as a co-receptor that mediates the communication of cancer cells by means of signaling mechanisms. Sarcoma formation may result from the activation of oncogenic mutations in cells expressing Ng2/Cspg4, such as mesenchymal progenitors [347].

Based on the low and restricted distribution of NG2/CSPG4 in normal tissues and its overexpression in several malignancies, there is emerging evidence of its role in cancer growth and dissemination. Thus, alteration in the expression and/or distribution of NG2/CSPG4 may serve as a prognosis in various cancer types [312, 383, 422]. Several preclinical studies have evaluated the role of targeting NG2/CSPG4 in soft-tissue sarcomas (STS) in which NG2/CSPG4 expression is correlated with tumor progression [24, 58, 279]. Regarding the functionality of NG2/CSPG4 in malignant progression, scientists proceeded to investigate whether it is overexpressed in STS, and whether their expression pattern could provide relevant information to use as a target for immune therapy. Inhibition of NG2/CSPG4 using antibodies has been reported to inhibit tumor growth in xenograft models [335, 422]. The confirmation of NG2/CSPG4 expression in triple-negative breast tumors (TNBC) might provide a therapeutic target for mAb-based immunotherapy [421]. However, the frequency and clinical significance of CSPG4 in breast cancer has yet to be determined.

1.4.4 Betaglycan

Betaglycan is a membrane proteoglycan containing heparan and chondroitin sulfate chains whose core protein binds to all three TGF-β isoforms, inhibin A, inhibin B, and certain bone morphogenetic proteins (BMPs). Betaglycan also exists in the soluble form, which presents distinct functions of the membrane form, since its purpose is to sequester ligands from their signaling receptors, thereby antagonizing signaling [28]. Notably, betaglycan is an important regulator of reproduction, fetal development and, in recent years, it has been identified as a tumor suppressor in many human cell types. It has been shown that downregulation or loss of betaglycan correlates with increased tumor progression. This loss of betaglycan can occur by mechanisms involving loss of heterozygosity at the betaglycan gene locus and epigenetic silencing [77].

The disrupted inhibin/betaglycan function is most strongly linked to ovarian cancers. Deletion of inhibin-α gene (INHA) results in granulosa and Sertoli cell tumors in the gonads and adrenal glands in mice, of both sexes [249]. In epithelial ovarian cancer cells, overexpression of betaglycan inhibited cell migration, while INHA gene silencing enhanced migration and invasion [149]. Betaglycan dowregulation has been associated with advanced-stage neuroblastomas, ovarian carcinomas, ovarian granulosa cell tumors, endometrial carcinomas, prostate cancer, renal cell carcinomas, non-small cell lung cancer, breast carcinomas, and pancreatic carcinomas [27]. It has also been shown that the expression of betaglycan in MCF-7 and MDA-MB-231 human breast cancer cells was able to restore TGF-β1-induced cell growth inhibition and reduced either anchorage-independent growth or tumorigenicity in athymic nude mice [63, 382].

The mechanism by which betaglycan regulates tumor cell growth, invasion and metastasis has not been fully elucidated. But the protection of betaglycan in tumors can be explained, at least in parts, by the inhibition of cell migration through the activation of small GTPase Cdc42, which is related to the organization of actin cytoskeletal and to the appearance of filopodial structures [271]. Betaglycan enhances the binding of all three TGF-β ligands to the TGF-β signaling receptors and it is essential for the high-affinity cell surface binding of TGF-β2. It also acts as a coreceptor for inhibin and it mediates signaling of different members of the BMP subfamily [27].

1.4.5 Phosphacan

Phosphacan is a splicing variant of the receptor-type protein-tyrosine phosphatase (RPTP), a nervous tissue-specific chondroitin sulfate proteoglycans (CSPGs). Phosphacan can be found in two forms, a secreted extracellular form and a short non-proteoglycan form [147]. It has already been demonstrated that the extracellular domain of RPTP and phosphacan binds to various cell adhesion molecules. However, little is known about the mechanism of signal transduction [330]. Phosphacan is implicated in axonal guidance and outgrowth in central nervous system development [179].

The role of phosphacan in cancer is still unclear, but it appears that its increase is associated with facilitating cancer progression and metastasis [362]. A possible mechanism for the action of phosphacan is that it can undergo glycosylation by the addition of O-mannosyl-linked HNK-1. This promotes the increase of β-catenin, which consequently retains RPTP-β/ζ on the plasma membrane and results in decreased cell adhesion and increased migration [301].

1.5 Intracellular Proteoglycans

1.5.1 Serglycin

The proteoglycan, serglycin, is an intracellular granule present in various cell types, such as macrophages, mast cells, neutrophils, platelets, cytotoxic T lymphocytes, and endothelial cells [206, 207], being a key mediator of granulopoiesis in these cells [139, 280, 430].

The size of serglycin may vary according to the number of GAG chains (CS4, CS6, CSE, CSB, or heparin [207]) attached to the protein

nucleus and to the variations in chain length of the connected GAGs [205], making their functions also vary [307, 349, 402, 440]. Its main chain can be divided into three demons: (1) signal peptide domain, (2) N-terminal domain, whose function is still unknown, (3) C-terminal domain [207].

Serglycin consists of a core protein containing a characteristic domain rich in serine/glycine repeats, in which it is linked to the chondroitin sulfate or heparin glycosaminoglycan chains (GAG), but it is negatively charged [208, 307], making it believed that this protein is important for homeostasis of positively charged components, such as proteases [1, 41, 150], and it is involved in the retention or secretion of histamine, cytokines, and chemokines in the storage granules of mast cells [150]. The release of tissue-type plasminogen activator from endothelial cells, tumor necrosis factor alpha (TNF-α) from macrophages, and matrix metalloproteinase 9 (MMP9) from monocytes are involved in the secretion of serglycin [349, 435, 464].

Heparin, a GAG with a remarkably high extent of sulfation and expressed by connective tissue mast cells, is the most well-known serglycin-associated GAG. Serglycin is replaced by lower sulfated chondroitin 4-sulfate (CS-4) chains in the various cells found in the circulation (lymphocytes, platelets, and monocytes) [205].

Although normal hematopoietic, endothelial, and embryonic stem cells showed mRNA or serglycin proteins [103, 349, 350, 402], it is also expressed in chondrocytes [467] and smooth muscle cells [217]. The interaction between serglycin and CD44 cell surface proteins has been demonstrated in both myelomonocytes and macrophages, as well as in lymphoma, myeloma, mastocytoma, and thymoma cells, in which serglycin attaches to the CS4 or CS6 moieties, but not to heparin or heparan sulfate [402]. High serglycin expression levels have been observed in multiple myeloma cells [392] and highly metastatic carcinomas [225]. It has been shown that a greater amount of serglycin was secreted by human breast CAFs [413]. In nasopharyngeal carcinoma and mammary carcinoma, an overexpression was observed [209, 225], and high serglycin levels were found in sera from patients with bone metastatic hepatocellular carcinoma [148], and in bone marrow aspirates from patients with multiple myeloma [321].

1.6 Collagens

Collagens are the most abundant proteins found in the animal kingdom being the major components of the ECM [101]. There are 28 different types of collagens that are found in fibrillar and non-fibrillar forms in vertebrates [134]. Whereas some collagens are important structural components in load-bearing tissues, others are essential elements of basement membranes. Collagens have a pivotal role in regulating cellular differentiation and pattern formation during embryogenesis and postnatal development [125]. The most common collagen in mammals is the fibrillar type I collagen, which is a principal component of interstitial matrices [34]. Type IV collagen is non-fibrillar and a key component of the basement membrane (BM) [181]. It is a network-forming collagen that underlies epithelial and endothelial cells and functions as a barrier between tissue compartments and is required for the maintenance of tissue polarity [102].

Increased synthesis of fibrillar collagens or perturbed turnover correlates with a variety of human diseases, including liver fibrosis, glomerulo-nephritis, vascular diseases, and tumor angiogenesis [270]. During tumor progression, many ECM proteins are significantly deregulated. The deposition of some proteoglycans and collagens is increased, leading to the reorganization of the tumor microenvironment. ECM remodeling destabilizes cell polarity and cell-cell adhesion, as well as increased signaling of growth factors, causing biochemical and biomechanical changes that together promote the metastatic cascade through cell migration into interstitial matrix and toward the vasculature [315]. In this way, collagens can act as a scaffold, facilitating migration of invading cancer cells or stromal cells [441].

The architecture of the collagen scaffolds in tumors is severely altered [270]. The collagen fibers surrounding the normal epithelial structures in soft tissues, for example, mammary gland and lung are typically curly and anisotropic. However, during tumor development, many of the fibers progressively thicken and linearize [219, 317]. These linearized fibers are stiffer than curly ones, so the increase in collagen density and concentration provides an increase in the stiffness of this matrix, causing disruption in mammary morphogenesis in 3D culture systems [303, 319], what results in a substantial growth factor-dependent cell migration into ECM [219, 463]. Notably, this linearization is major in regions adjacent to the tumor vasculature and in areas with cancer invasion [74, 219, 317].

Since cell-ECM interaction is an integral part of cancer progression, collagen cross-linking and matrix rigidity in vivo are correlated with lysyl-oxidative enzyme (LOX) replication, which in addition to being associated with a poor prognosis, is also involved in the recruitment of stromal cells [219]. Increased matrix stiffness causes an increase in tension, which induces integrin grouping, development of focal adhesions, and activation of multiple downstream signaling pathways, including phosphorylation of focal adhesion kinase (FAK) and Cas [219]. Activation of FAK may increase the phosphorylation of extracellular signal-regulated kinase (ERK) via Ras activation [303, 319]. ERK can control migration, invasion, proliferation, and cell differentiation through the modulation of myosin contraction, as well as the induction of transcription programs [319]. This interaction occurs through the activation of ECM receptors, including the 24 human integrins, which constitutes the dominant class of ECM receptors [92], for example, discoidin domain receptor (DDR) [418], CD44 and CD36 [180].

Increased tissue density is often observed in malignant breast cancer, and growing evidence suggests that high tissue tension may significantly influence cancer progression [219, 318]. In addition, the increased risk of breast cancer has been associated with high tissue density, and studies using animal models suggest that this link has a causal significance [39, 318]. Studies with mouse models have shown that changes in the interstitial matrix and increased stiffness may be induced by obesity and may promote mammary tumorigenesis [351].

The excessive production of collagens is a common feature of fibrosis [27] and malignant breast cancer, in which there is accumulation of fibrillar collagens I, III, and V, and decreased quantities of type IV [102, 233, 298] due to degradation of the basement membrane, which is also a common feature of the involuting mammary gland [233, 298]. In human breast tumors, the increased risk of metastatic recurrence is related to certain collagens [327, 386], for example increased expression of type I and III may be linked to tumor invasion and aggressive behavior [144, 185].

1.7 Elastin

Elastin is the main component of elastic fibers being formed through multimerization and cross-linking of tropoelastin monomers in the presence of elastic fiber proteins. Elastin fibers are made up of two components: an amorphous component, consisting of elastin, and microfibrils that act as a scaffold for elastin incorporation [99]. Because elastin deposition is performed only in the early stages of life, it can be said that it does not change in volume, and it is assumed that each individual has a certain amount of this protein that should last the rest of life [343].

Over the years elastin undergoes fragmentation, resulting in the release of elastin-derived peptides (EDPs). These EDPs display a wide range of biological activities, influencing cell migration, differentiation, proliferation, chemotaxis, survival, tumor progression, angiogenesis, aneurysm formation, and atherogenesis [94, 343]. It has been demonstrated that EDPs increase with B16F1 melanoma development, and it is induced during in vitro proliferation of tumor cells [93]. It has also been demonstrated that the presence of EDPs can promote tumor invasion of fibroblastoma [94], lung carcinoma [401], and glioblastoma [78] cells. The presence of EDPs enhances matrix invasion of human breast cancer

cells (MDA-MB-231) and promotes the secretion and activation of MMP-2 [343]. The combination of deposition and degradation of elastin promotes the formation of a complex phenomenon called elastosis [189].

1.8 Fibronectin

Fibronectin (FN) is a fibril-forming glycoprotein that is assembled into a fibrillar matrix in all tissues and throughout all stages of life. FN has domains that allow a wide variety of cellular interactions with other ECM proteins, cell surface receptors, and glycosaminoglycans (GAGs) [161, 244] and plays important roles in cell adhesion, migration, growth, and differentiation [55, 72, 448]. Some studies indicate that FN is required for collagen incorporation into the ECM [368]. The increasing interest in FN is due to its participation in the promotion of tumor growth in multiple stages of tumor progression, facilitating tumor cell invasion and migration, which may be due to mechanical compression [142, 188, 203, 234, 258, 262, 273, 400, 405, 425, 429, 454].

On the early stages of cancer development, there is infiltration of populations of immune cells, which despite being primarily tumor suppressive, may undergo phenotypic changes that promote the dissemination depending on the presence of accessory stromal cells and local cytokine milieu [89]. In mouse models, the expression of FN has been reported to be induced in the metastatic niche by cytokines secreted from the primary tumor, which generates a stromal niche conducive to metastasis [183].

In breast tumor stroma, FN mRNA and protein levels were observed, although they are not expressed in normal adult breast tissue [166]. Some studies indicate that the FN derived from cancerous cells in primary mammary tumors present invasive and metastatic phenotypes [470], as well as being associated with survival and clinical outcome [13, 110]. Furthermore, circulating tumor cells (CTCs), which are cells that have shed into the vasculature or lymphatics from a primary tumor and are carried around the body in the blood circulation, also express FN [325]. During the epithelial-mesenchymal transition (EMT), the CTCs that are still stuck can leak, and consequently, remain inactive (dormancy) or insert and promote colonization. In the EMT process, the cells lose polarity and cell–cell adhesion observed in epithelium acquire a mesenchymal phenotype including motility, invasiveness [461]. FN, which is an established mesenchymal marker, has been shown to promote TGFβ-induced EMT [302]. Indeed, FN-positive CTCs express several EMT markers [325].

FN, like collagen, is strongly upregulated during fibrotic response or desmoplasia, in which myofibroblasts within the tumor stroma produce an organized fibrotic ECM rich in FN and type I collagen [224, 287]. The combination of fibronectin and type I collagen in the tumor stroma is associated with poor prognosis [32].

The modulatory effects of FN on the signaling pathways of cancer cells occur through the induction of the signal transducer and activator of transcription 3 (STAT3), which is a transcription factor that regulates cell proliferation and survival. It also functions as a major player inducing the growth of stem-like breast cancer cells [163, 245]; as well as MAPK pathway, promoting invasion and metastasis in model systems [16, 323]. FN also participates in the regulation of cellular responses stimulated by IGFBP-3 and EGF. Thus, in the presence of FN, treatment with IGFBP-3 and EGF promotes growth, whereas in its absence these growth factors inhibit growth [255].

1.9 Laminins

Laminins form a group of large heterotrimer glycoproteins. They represent the main non-collagenous proteins of basement membranes that constitute the extracellular matrix proteins and are involved in multiple important biological activities. They modulate several cellular homeostatic functions in normal cells which are often found deregulated in carcinomas [106, 141, 242], such as assembly of the basement membrane [242], cell attachment [242, 243], migration [12, 242], growth and differentiation [128, 242] and angiogenesis [191, 242]. Twelve different laminin

forms are known that have cell- and tissue-specific expression and are recognized by integrins and other receptors [304]. Several laminin isoforms play significant roles in breast cancer development. In the basal membranes derived from embryonic tissues, laminin-111 (LM-111) is present, which is a large molecule associated with multiple biological activities. It binds to the other abundant basement membrane components, including collagen IV, perlecan, entactin/nidogen, and itself. The interaction with cells promotes multiple biological activities, such as cell adhesion, migration, neurite outgrowth, tumor growth, and metastasis [104, 194, 201]. Mutated mammary epithelial cells, during the onset of breast cancer, fail to respond to signals from the basal membrane [310], then activate degradation of LM-111 by matrix metalloproteinases disrupting the architecture of mammary glands acini, reinitiating cell proliferation [23] and leading to eventual formation of mammary tumors [29].

LM-111 is secreted by the normal myofibroblasts of the breast and maintains the epithelial polarity. It is also responsible for promoting prolactin-induced maturation of breast epithelial cells [376, 377]. The change in cell polarity is associated with the loss of LM-111 expression in breast tumors [141]. The decrease in the expression of DNA methyl transferase-1 (DNMT1) prevents the methylation of the E-cadherin promoter, which increases its expression levels, causing induction of cell-cell adhesion, and this suggests that LM-111 may act as an inhibitor of the spread of breast cancer in three-dimensional cultures [25].

There is evidence that indicates that other laminins may promote tumor progression. LM-332 is a major adhesive component of the epidermal basal membrane [56, 339]. It promotes attachment, spreading, scattering, and migration of non-tumorigenic epithelial cells by interacting mainly with integrin α3β1 at far lower concentrations than other cell adhesive proteins [193, 338]. The interaction of LM-332 with integrin α3β1 or α6β4 induces intracellular signal transduction to support cellular survival, proliferation, and migration by activating many signal mediators [184, 278, 284]. There are studies showing that these two LM-332 receptors (integrins α3β1 and α6β4) are associated with the malignant behavior of tumor cells [129]. The interaction between integrin α3β1 and vascular LM-332 mediates pulmonary arrest and metastasis [420]. The anchorage-independent survival through the interaction with the receptor of α6β4 integrin is promoted by cell-derived LM-332, which is associated with aggressive breast cancer [215, 462]. This molecular mechanism is mediated by the Rho family GTPase RAC and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [462]. The induction of migration and invasion of breast cancer cell are due to the LM-332 via α3 integrin [54]. A high EMT activity in human breast cancer samples was observed and associated with LM-332 expression at the boundaries of normal breast and tumor tissues [197]. In the co-culture of breast cancer cells with primary fibroblasts from the mammary gland, there was an induced LM-332 and integrin β4 expression, which promoted cell resistance to anoikis [199].

High expression of LM-511 subunits has been reported in many cancers [42, 66, 122, 151, 186, 238, 293, 364] and high to moderate expression has been shown in fibroadenoma, ductal carcinoma in situ (DCIS), tubular carcinomas, atypical medullary carcinomas, and carcinomas of no specific types [151, 238]. However, there is a significant variation in the precise pattern of LM-511 expression according to tumor types, and its level in the tumor or in the associated vasculature is influenced by the stage of the tumor [42, 66, 118, 122, 186].

In experimental breast cancer, LM-511 has been shown to mediate adhesion, migration, and invasion, and in vitro it promotes metastasis via integrin interaction [66, 214]. The interaction of LM-511 with α6β1 integrin receptors, in a subpopulation of breast cancer cells, promotes self-renewal and tumor-initiating capabilities [61], resulting in increased TAZ activity and subsequent regulation of LM-511, which culminates in increased ability of cells to form mammospheres, characteristically enriched in stem cells [61].

α4-laminins, such as 411, 421, and 423, are produced by vascular and several other cell types of mesenchymal origin [120, 121, 124, 143, 227, 248, 259, 306, 308, 414, 437]. Laminin-411 is recognized by some integrins and promotes adhesion and/or migration of several cells as well as neurite outgrowth [120, 124, 133, 210, 226, 306, 385, 437]. The increase in clonal expansion of breast cancer cells and integrin β1-dependent tumor re-initiation due to α4 laminin encoded by the LAMA4 gene and a subunit of LM-411 and LM-421 was observed in multiple organs in a mouse model of breast cancer [337]. FOXQ1 transcription factor induces LAMA4 expression, and poor outcome in breast cancer patients was associated with LAMA4 [337].

1.10 Matricellular Proteins

Cell-matrix interactions as well as cellular functions are modulated by matricellular proteins, which are extracellular matrix proteins that do not play a direct structural role. They are composed of a group of structurally diverse ECM glycoproteins, such as osteopontin, tenascins, periostin, SPARC, and thrombospondins. A common property of matricellular proteins is their high expression during embryogenesis, which strongly decreases after birth, when expression becomes low to absent during adult life and is primarily associated with stem cell niches and tissues undergoing remodeling such as wound healing and inflammation [268, 298]. The modulation of cell-matrix interaction is carried on through interaction with cell-surface receptors, proteases, hormones, and other bioeffector molecules, as well as with structural matrix proteins such as collagens and fibronectin. The interaction between these proteins may antagonize the adhesive properties of other MEC proteins, which may lead to an intermediate pattern of adhesion [267].

The heterogeneous group of matricellular proteins has, as common feature, the anti-adhesive properties [268]. Although the matricellular proteins have the ability to bind to structural components of the ECM to perform structural functions, such as collagens, they are not believed to contribute significantly to the formation of the ECM structure. However, there is evidence that they play an important role as cellular regulators and modulators of signaling pathways [69, 438]. Many of these proteins have also been implicated in tumor development and progression [344]. During tumor progression, cancer cells demand dynamic cell adhesion attributes, and high expression of matricellular proteins in tumors has been observed. Increase in the expression of these proteins is commonly associated with metastatic spread and poor outcome in cancer patients [69]. Cellular motility is modified due to changes in cell adhesion, and most of the matricellular proteins are related to increased motility and invasive cancer cell behavior. However, other cellular functions modulated by the matricellular proteins, such as survival and growth under stressful conditions, are highlighted [241, 298].

1.10.1 Osteopontin

Osteopontin (OPN), also called secreted phosphoprotein 1 (SPP1), is a matricellular protein highly expressed during embryonic development [127, 389]. OPN is expressed in low levels during the postnatal life depending on the tissue [91]. OPN expression increases during pathological conditions, such as cancer [408], indicating its role in matrix remodeling and cell-matrix interaction in diseased tissue. It is a phosphorylated glycoprotein that binds integrins and functions as a mediator of cell adhesion, migration, immune response, and tissue repair [51, 371, 423].

Elevated levels of OPN have been found in several tumors [4, 18, 115, 165, 228, 409, 469]. In breast cancer, OPN has been linked to increased progression [354, 407], poor prognosis [410, 427], and increase in cancer cell survival and migration [60, 355].

Postnatally during pregnancy and lactation, mammary gland epithelial cells grow and undergo differentiation [239]. Some studies

analyzed the expression pattern and possible role of OPN in normal mammary gland development [15, 277, 334]. OPN is found in milk, plasma, urine, and other bodily fluids [91]. In normal mammary glands, SPP1 expression is generally low, although it is induced during lactation and involution [334]. Increased proliferation and altered differentiation of mammary epithelial cells was observed in transgenic mice expressing SPP1 [160].

Several studies have suggested a correlation between OPN levels and the progression of cancers [4, 388]. It is believed that SPP1 expression alone is not enough to generate tumors; however, some results suggest that SPP1 may be correlated with the progression of mammary tumors. Indeed, in a rat model, SPP1 expression has been shown to promote metastasis in non-metastatic mammary tumors [291]. In genetic mouse models, in which mammary tumors are driven by expression of c-myc and v-Ha-ras oncogenes, SPP1 was not required for tumor development, indicating that it is important, particularly in the later stages in tumor progression and metastasis, or that is dependent on a more specific context [109]. In mouse models, breast cancer cells with increased ability to metastasize to bones exhibited higher SPP1 expression when compared to the primary tumor cells; this allows for the interpretation that SPP1 expression is linked to increased metastasis [182].

Cancer biomarkers have been used to predict patterns of the disease. In this way, the progression of breast cancer in humans could be predicted using SSP1 as a biomarker. Expression of SPP1 in breast cancer samples from a cohort of lymph node-negative patients has been shown to be associated with poor relapse-free and overall survival [409]. Moreover, when plasma levels of SPP1 were quantified by enzyme-linked immunosorbent assay (ELISA), there was a correlation between increased tumor burden and decreased survival of breast cancer patients [357]. Although high SPP1 levels in serum may reflect its expression in tumors from different sites, there is evidence that SPP1 in serum has functional roles involving the mobilization of stromal cells from bone marrow [254].

1.10.2 Tenascin C

Tenascin C (TNC) is a large, multifunctional ECM glycoprotein with a hexameric structure able to interact with cell surface receptors and other ECM proteins, such as fibronectin, periostin, integrin cell adhesion receptors, and syndecan membrane proteoglycans [192, 297]. It has been shown that TNC promotes cell migration [313], inhibits focal adhesion formation [269], induces cell proliferation [71], promotes angiogenesis [348], and can induce expression of matrix metalloproteinases [403], which themselves have been implicated in promoting tumor growth and invasion [374].

During the development of bone and cartilage, and in neural crest cells, TNC is abundantly expressed. However, in adults, TNC is present in stem cell niche regions and in sites of epithelial-mesenchymal interaction. In mechanical stress and inflammation during wound healing, as well as in tumor-associated connective tissue, TNC expression is elevated [297, 419].

Studies have described changes in the profile of TNC isoforms expressed in tumors, such as breast, lung, colorectal, ovarian carcinomas, and glioblastomas [37, 53, 97, 152, 434], when compared to normal tissues. The alteration in cell adhesion and motility, which may promote invasion and metastasis, is influenced by TNC. It can also influence the expression of tumor suppressor genes, oncogenes, and genes involved in the maintenance of genomic stability [67, 297]. Changes in the epithelial-mesenchymal transition (EMT) can also be induced by TNC, leading to loss of intercellular adhesion and increased migration of breast cancer cells [272]. Induction of TNC expression is induced by transforming growth factor β (TGF-β) [178].

In the mammary gland, TNC expression is associated with gland involution and breast cancer development [68]. There is evidence indicating that TNC expression in breast tumors is a predictor of metastatic relapse and poor overall survival [166, 173, 299]. This link was confirmed through experimental analyses that demonstrated the functional role of TNC in breast cancer progression. TNC expression in breast cancer cells is

part of a gene signature associated with metastasis to lung, in addition to being regulated by microRNA miR335 [260, 386], in which metastatic cells with a high propensity to colonize the lung lose expression of miR335, causing upregulation of TNC [299, 386]. The increase in survival and metastatic capacity that occurs in TNC-mediated lung metastasis is due to the Wnt and Notch pathways [299]. The ability of metastatic breast cancer cells to colonize lungs and bones is decreased when there is knockdown of TNC, which indicates the need for autocrine TNC in metastasis [299].

For the colonization of distant organs by cancer cells, the autocrine TNC is necessary, however, the stromal-derived TNC also supports metastatic colonization. Mammary carcinoma cells implanted into mammary fat pads of TNC knockout mice generate significantly fewer lung metastases compared to control mice [290]. Although cancer cell-derived TNC is required for the initial stages of metastatic colonization, it is not essential at later times when the activated tumor stroma becomes a considerable source of TNC [299]. Tenascin-C has been reported in the early stages of prostate carcinogenesis, being a hallmark of the reactive stroma [412].

1.10.3 Periostin

Periostin (POSTN) is a homodimeric matrix protein that is preferentially expressed in the periosteum, its activity is elevated during the embryonic development and body growth phases, although during adult life it contributes to bone resistance [33, 257]. Embryonic fibroblasts and pericardial cells covering the embryonic heart show high expression of periostin, suggesting its important role in cardiac development and tissue repair in the adult individual [164, 340, 363]. POSTN is also expressed in other connective tissues rich in mechanical stress collagen such as the periodontal ligament [154], heart valves [211], and tendons [458].

POSTN is known to interact with integrin receptors [153], with other ECM proteins [192, 288], and modulates intracellular tyrosine kinase signaling and focal adhesion kinase (FAK) [231]. Due to these findings, it is suggested that POSTN may regulate collagen fibrillogenesis and the biomechanical properties of connective tissues through the formation of reticular structures [192, 288]. Thus, POSTN promotes the organization of the ECM to support invasion and metastasis, since alterations occur in the ECM components of the tumor microenvironment [212].

In some pathological conditions, such as inflammation [231], tissue repair [296], and malignant transformation [36, 329], the expression of periostin appears deregulated. Although POSTN is involved in cancer initiation, progression, and metastasis, its high levels are associated with more aggressive tumor behavior, advanced stage or poor prognosis, and which can be used as a possible biomarker [398]. In many cancer types, such as non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and other [17, 20, 83, 365], POSTN levels are upregulated. However, there are reports describing POSTN as a tumor-inhibiting factor in bladder cancer and osteosarcoma [195, 459].

Although POSTN expression has been observed in the end buds of mammary glands [241], it has been suggested that it might not be necessary for mammary gland development in null mice [370]. Together with the evidence that POSTN plays an important role in breast tumor progression and metastasis, it has been observed that overexpression in human breast cancer cells induced primary tumor growth as well as angiogenesis in a xenograft model [320, 352, 466]. It has been shown that POSTN also interacts with decorin, which is a potential natural anticancer agent produced by normal cells [345], and this interaction forms intracellular complexes that prevent decorin from being secreted into the extracellular space [172]. Decreased in vitro motility and invasiveness were observed in invasive breast cancer cells submitted to POSTN knockdown. These cells also exhibited induction in decorin secretion [172].

Metastatic bone tumors, besides being the most common type of malignant bone lesions observed in adults and the third most frequent

metastatic site after the lung and liver [455], are also the main causes of increased morbidity and eventual mortality in patients with breast cancer (75% in women).

High POSTN expression in the stroma around metastatic tumors and high POSTN plasma levels were observed in murine xenograft model in mice bearing bone metastasis [75]. POSTN displayed to be a key ECM component of the metastatic niche that supports the metastasis-initiating cells with stem-like properties [241]. POSTN ablation in the stroma of null mice for this gene has significantly impaired lung metastatic ability of the tumors, although such mice have developed mammary tumors directed by the medium polyoma T transgene (PyMT), the effect of which has been linked to improve signaling Wnt and stimulate the ability to initiate breast cancer cell metastases [241]. In metastasis, cancer-fibroblast cells (CAFs) and germinal endothelial cells were found to be sources of POSTN [126, 241]. It is overexpressed in cancer stem cells population (CSCs) that expresses high CD44 and low CD24 surface markers and is downregulated by siRNA-sensitized breast CSCs [447]. POSTN binds to TNC, which is incorporated in the ECM composed of type I collagen and fibronectin [192], suggesting that this collaboration leads to the formation of the metastatic niche, where POSTN presents Wnt ligands to the cancer cells and TNC regulates the capacity of these cells to respond to Wnt [298].

1.10.4 SPARC

Matrix remodeling and cell motility are modulated by secreted protein acidic and rich in cysteine (SPARC), also termed as osteonectin or basement-membrane protein 40 (BM-40). It is the major noncollagenous protein of bone matrix [387] that plays a vital role in bone mineralization, cell-matrix interactions, and collagen binding. SPARC is a glycoprotein which function and structure are modulated by Ca^{2+} ions. It exhibits several functions that regulate cell shape and proliferation and regulate changes in the organization of the extracellular matrix [40, 341]. Events that require change in cell shape and motility, for example, tissue renewal, tissue remodeling, and embryonic development, often express these proteins [35, 332].

SPARC's best-characterized interaction is to collagens in a Ca^{2+}-dependent manner. There is a greater affinity of SPARC for collagen IV, which is the most abundant collagen in the basal lamina [346], although SPARC also binds with similar affinity to fibrillar collagens type I [171, 387], II and III [342], IV [252, 253], V and VIII [342], which could result in the remodeling of ECM according to the specific interaction. There is a change in the affinity of SPARC for collagen when it is bound to calcium and causes an increase in α-helicity, which leads to a change in conformation that reduces the susceptibility of the EC domain to proteinases [105, 251, 316]. Studies have shown that SPARC also binds to VEGF and that this interaction interferes with the binding of VEGF to human microvascular endothelial cells, leading to a reduction in the association of VEGF with its Flt-1 cell surface receptor. Thus, SPARC inhibits endothelial cell proliferation induced by VEGF [213]. It also has been demonstrated the co-localization in vivo of vitronectin and SPARC and interaction via the heparin-binding region with the C-terminal EF-hand of SPARC [336]. Part of the differential morphoregulatory processes required of cells in the remodeling tissues can be explained by the report of the opposing effects on cell adhesion caused by these two proteins [256].

Due to the diverse functions of SPARC in the microenvironment, it appears to be cell-type specific which leads to a complexity of its role in tumorigenesis. Both the SPARC protein isolated from the basal membrane of the tumor and recombinant human SPARC have been shown to bind to the basement membrane in the presence of calcium [253, 286]. The level of SPARC transcript is higher in breast tumors than in normal breast tissue [426], and it is increased in tumors from patients with high-grade invasive breast cancer [426]. Poor clinical outcome, increased recurrence of early breast tumors, and poor overall survival in patients with invasive breast cancer have been linked with the SPARC's expression

[156, 436]. A xenograft mouse model has shown that SPARC is sufficient and required for the development of lung metastasis [260]. Although the expression of SPARC can be used to determine the malignant behavior of some cancers, in others it is described as a tumor suppressor [116].

1.10.5 Thrombospondins

The thrombospondins (TSP) are a family of high-molecular-weight glycoproteins that are secreted by almost all of the cell types. In mammals, TSPs have many specific complex roles, among them, wound healing and angiogenesis, vessel wall biology, connective tissue organization, and synaptogenesis, due to their interaction with cell surface, growth factors, cytokines, or ECM components which regulate many aspects of cell phenotype [2]. TSP constitute a five-member family of multimeric glycoproteins that bind to Ca2 and interact with other ECM proteins contributing to cell-cell and matrix-cell associations [2]. They are widely expressed during vertebrate development and in various diseases including cancer, osteoarthritis, and muscular dystrophies [250, 415, 465].

The first TPS identified (TSP1) is the most studied thrombospondin because of its involvement in cell adhesion and migration; it has functional activity in the control of angiogenesis, in addition to being associated with wound healing and cancer. It also regulates matrix structure, extracellular proteases, levels of active TGF-β [2], and cellular phenotype [187, 375]. TSP1 via activation of TGF-β and upregulation of the urokinase plasminogen activator system may induce invasion of cancer cells [5, 246].

TSP1 is a molecule found naturally in vertebrates that can inhibit angiogenesis [38], presenting an inhibitory effect on cellular migration in vascular endothelial cells [64, 86]. The binding of TPS1 or TPS2 to the CD36 receptor on endothelial cells causes inhibition of angiogenesis and induces apoptosis [2]. In addition, during the development of malignant tumors, TSP1 may operate as an antiangiogenic barrier [84, 119, 157]. In breast cancer models, TSP1 antiangiogenic function leads to inhibition of primary tumor growth [453]. Nonetheless, TSP-1 expression in tumor samples or high levels in plasma from breast cancer patients is associated with poor relapse-free survival [114].

TSP1 functions as a suppressor in immune regulation through its interaction with CD47 on immune cells by directly affecting dendritic cells and T cells [76, 222, 223]. However, it promotes migration in neural crest cells [411], fibroblasts [137], corneal endothelial cells [31], and vascular smooth muscle cells [64]. TPS1 expression regulates angiogenesis and tumor progression; it inhibits the proliferation of some tumor cells [428] while promoting the proliferation of others [113, 289]. This fact is due to the downstream functions influenced by TSP1 in tumor cell migration [6].

Whereas the level of TSP1 expression is decreased in many tumor cells, strong expression of TSP1 is associated with the tumor stroma [46]. In general, the expression of TSP-1 is decreased in tumor cells that contain mutations affecting oncogenes and tumor suppressor genes [87] and has been shown to occur in cells with variations in the levels of hRAS, Jun, Myc, vScr, and p53 [130]. In clinical melanoma, bladder carcinoma and colon carcinoma samples decreased TSP1 correlates with mutation in p53 [138, 399]. Moreover, studies in mouse models have shown that TSP1 and 2 indeed promote metastatic colonization of distant organs [90, 453]. TSP2 expression by cancer cells promotes stromal activation in secondary organs which fuels metastatic colonization [90].

1.11 Interactome

Proteins are biomolecules that facilitate the most of biological processes: gene expression, cell growth, proliferation, nutrient uptake, morphology, motility, intercellular communication, and apoptosis [145, 159, 230, 232, 295, 300, 379, 450].

In biological processes the protein-protein interactions are essential, and it can occur at inter- and intracellular levels [11]. Biological interaction networks, such as protein-protein

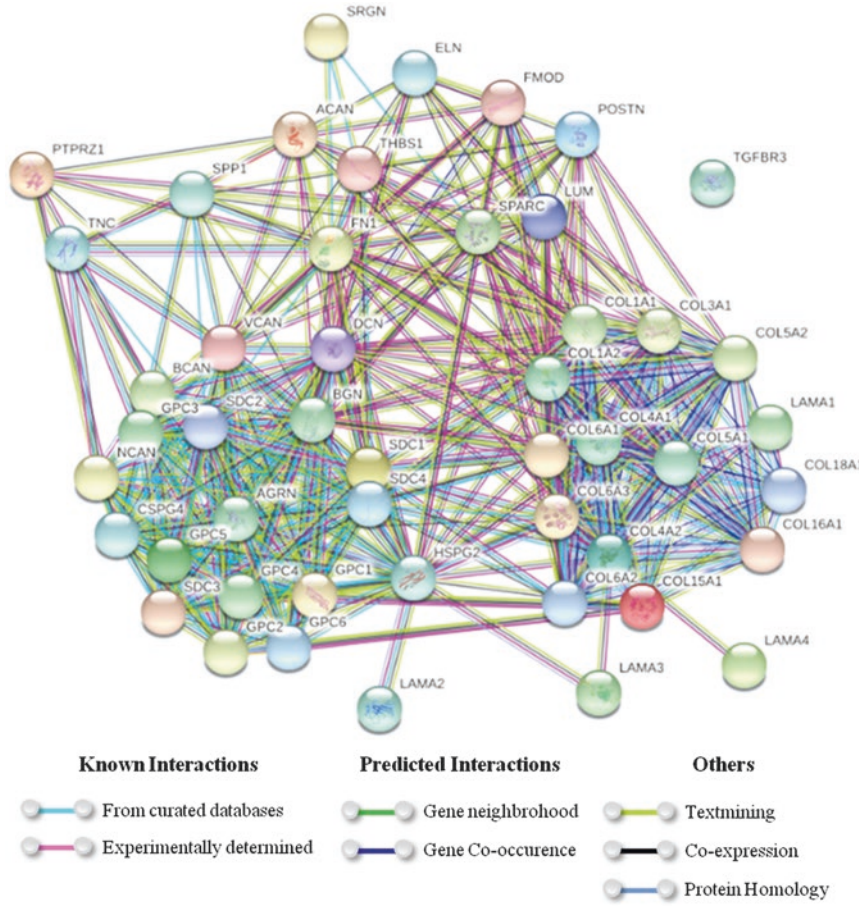

Fig. 1.3 Interactome of proteins present in the extracellular matrix. The interactome was generated using STRING program version 11.0 (string-db.org). The STRING interface uses merged data from different databases to construct protein interaction outlines. The network was connected by using the proteins described in the text of the review. ACAN binds to HA. AGRN modulates different growth factor signaling pathways. BCAN stabilizes interactions between HA and brain proteoglycans. Biglycan interacts specially with collagen promoting the assembly of collagen fibers. CSPG4 binds collagen alpha 2(VI) to the cell surface. Decorin (DCN) influences the formation of fibrils. Elastin (ELN) is usually associated with other proteins, such as collagens, in or colocalized with microfibrils, and bind to elastogenic cell surface receptors. Fibromodulin (FMOD) affects the rate of fibril formation, playing a major role in collagen fibrillogenesis. Fibronectin (FN1) binds cell surfaces to various components of the extracellular matrix. Perlecan (HSPG2) is an integral component of basement membranes, composing the glomerular basement membrane (GBM). Lumican (LUM) is related to collagen organization. NCAN binds to HA. Periostin (POSTN) enhances the incorporation of bone morphogenetic protein 1 (BMP1) in the fibronectin matrix of connective tissues, and subsequent proteolytic activation of lysyl oxidase (LOX). Phosphacan (PTPRZ1) can interact with tenascin C. Serglycin (SRGN) participates in the regulation of secretory vesicles. It is also required for the storage of some proteases and mediates the processing of MMP2, plays a role in cytotoxic cell granule-mediated apoptosis and regulates the secretion of TNF-α. SDC1 binds the cytoskeleton to the interstitial matrix, whereas SDC2 is present in the cell membrane and regulates cell proliferation and migration binding to ECM proteins. Different tumor types demonstrated altered syndecan-2 expression. SDC3 may affect the actin cytoskeleton and regulate cell shape. Secreted protein acidic cysteine-rich (SPARC) binds to calcium and copper; several types of collagen, albumin, thrombospondin (THBS1), platelet-derived growth factor (PDGF), and cell membranes. Osteopontin (SSP1) binds tightly to hydroxyapatite, appearing as an integral part of the mineralized matrix; besides being important to cell-matrix interaction. THSB1 is a glycoprotein that mediates cellular interactions to other cells as well as to the matrix. Tenascin C (TNC) is a high-affinity ligand for aggrecan, versican, and brevican as well as for neurocan. VCAN participates in cellular signaling and in cellular connections to the ECM, besides binding to HA

interaction (PPI) and protein-DNA interaction networks (also called gene regulatory networks), can be demonstrated in interactomes [146]. Interactomics uses the intersection of bioinformatics [261] and biology [424] to study the interactions between proteins and other molecules belonging to different biochemical families, as well as within a given family, such as nucleic acids, lipids, and carbohydrates [9, 44, 202]. Interactomes can reveal important interactions between molecules that preliminarily had no functional relationship and can provide practical drug targets for oncology drug discovery [70, 369]. The relationships between proteins described above are shown by the interactome contained in Fig. 1.3.

1.12 Conclusion

In summary, it is observed that in every stage of tumor progression multiple extracellular proteins are present, and seem to be actively involved. Therefore, ECMs have an increasingly important role in the pathogenesis of cancer, and this leads us to believe that the detailed understanding of their role in the tumor microenvironment may be key in the discovery of new anti-tumor therapies. In this book, the main ECM components will be discussed in more detail in each chapter.

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Keratan Sulphate in the Tumour Environment

2

Anthony J. Hayes and James Melrose

Abstract

Keratan sulphate (KS) is a bioactive glycosaminoglycan (GAG) of some complexity composed of the repeat disaccharide D-galactose β1→4 glycosidically linked to N-acetyl glucosamine. During the biosynthesis of KS, a family of glycosyltransferase and sulphotransferase enzymes act sequentially and in a coordinated fashion to add D-galactose (D-Gal) then N-acetyl glucosamine (GlcNAc) to a GlcNAc acceptor residue at the reducing terminus of a nascent KS chain to effect chain elongation. D-Gal and GlcNAc can both undergo sulphation at C6 but this occurs more frequently on GlcNAc than D-Gal. Sulphation along the developing KS chain is not uniform and contains regions of variable length where no sulphation occurs, regions which are monosulphated mainly on GlcNAc and further regions of high sulphation where both of the repeat disaccharides are sulphated. Each of these respective regions in the KS chain can be of variable length leading to KS complexity in terms of chain length and charge localization along the KS chain. Like other GAGs, it is these variably sulphated regions in KS which define its interactive properties with ligands such as growth factors, morphogens and cytokines and which determine the functional properties of tissues containing KS. Further adding to KS complexity is the identification of three different linkage structures in KS to asparagine (N-linked) or to threonine or serine residues (O-linked) in proteoglycan core proteins which has allowed the categorization of KS into three types, namely KS-I (corneal KS, N-linked), KS-II (skeletal KS, O-linked) or KS-III (brain KS, O-linked). KS-I to -III are also subject to variable addition of L-fucose and sialic acid groups. Furthermore, the GlcNAc residues of some members of the mucin-like glycoprotein family can also act as acceptor molecules for the addition of D-Gal and GlcNAc residues which can also be sulphated leading to small low sulphation glycoforms of KS. These differ from the more heavily sulphated KS chains found on proteoglycans. Like other GAGs, KS has evolved

A. J. Hayes
Bioimaging Research Hub, Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, UK

J. Melrose (✉)
Graduate School of Biomedical Engineering, University of New South Wales, Sydney, NSW, Australia

Raymond Purves Laboratory, Institute of Bone and Joint Research, Kolling Institute, Northern Sydney Local Health District, Royal North Shore Hospital, St. Leonards, NSW, Australia

Sydney Medical School, Northern, The University of Sydney, Faculty of Medicine and Health at Royal North Shore Hospital, St. Leonards, NSW, Australia
e-mail: james.melrose@sydney.edu.au

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molecular recognition and information transfer properties over hundreds of millions of years of vertebrate and invertebrate evolution which equips them with cell mediatory properties in normal cellular processes and in aberrant pathological situations such as in tumourogenesis. Two KS-proteoglycans in particular, podocalyxin and lumican, are cell membrane, intracellular or stromal tissue–associated components with roles in the promotion or regulation of tumour development, mucin-like KS glycoproteins may also contribute to tumourogenesis. A greater understanding of the biology of KS may allow better methodology to be developed to more effectively combat tumourogenic processes.

Keywords

Keratan sulphate · Sulphation motifs · Tumour marker · Podocalyxin · Phosphacan · Keratocan · KS mucin glycoproteins · KS antibodies, 5-D-4, 1-B-4, MZ-15, 4C4, R-10G, D9B1 · SV2 proteoglycan · Aggrecan · Astrocytomas

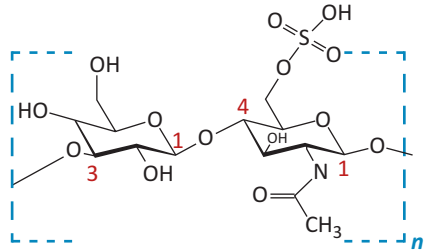
2.1 Introduction

Glycosaminoglycans (GAGs) have evolved over hundreds of millions of years of vertebrate and invertebrate evolution through positive evolutionary selection pressures, resulting in the positive selection of GAGs with an ability to participate in a diverse range of essential physiological processes [1, 2]. GAGs are sophisticated biodiverse components of the glycocalyx surrounding all cells and convey molecular recognition and structural information important in cellular regulation and tissue homeostasis [3–8]. While GAGs are composed of regular repeat disaccharides, it is the non-uniform sulphation patterns along the GAG backbone which have important extracellular matrix and cell regulatory properties. These sulphation patterns are the functional determinants on GAGs that equip them with interactive properties with extracellular matrix (ECM) components, growth factors, morphogens and cytokines and allow them to regulate tissue development/remodelling and the maintenance of tissue homeostasis in health and disease [3, 9–12]. Significant alterations in GAG distributions and composition have been noted in a number of tumours; these are of diagnostic value and tumour secretions containing these GAGs have proven useful as biomarkers of the pathological status of tissues and the degree of tumour development or regression following therapeutic intervention [13, 14].

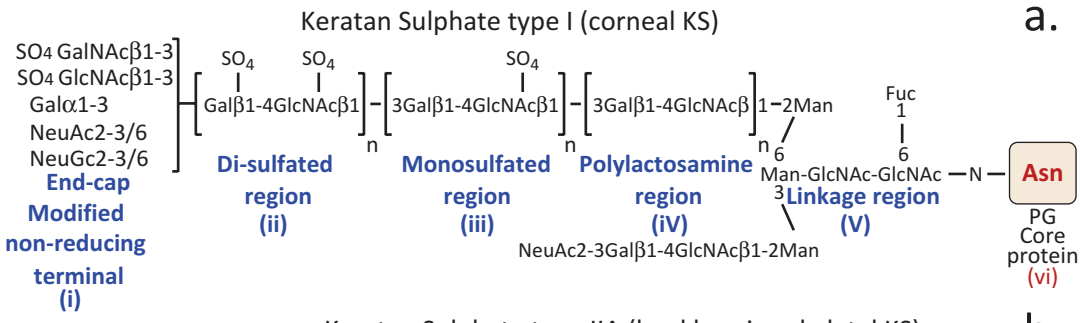
2.2 Keratan Sulphate Structure

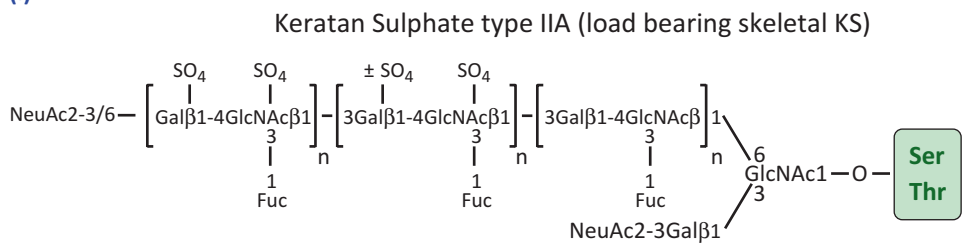
Keratan sulphate (KS)[1] is a GAG which has a widespread distribution in connective tissues [17, 18]. KS is composed of the β1-4 glycosidically linked repeat disaccharide Gal-GlcNAc which are sulphated at C6 either individually or collectively, leading to regions of mono- or disulphation in the KS chain; regions of non-sulphation have also been identified referred to as (poly) N-acetyl lactosamine regions in the KS chain although a number of proteins also contain lactosamine (Fig. 2.1). The linkage region at the reducing terminus of the KS chain to proteoglycan (PG) core protein acts as an acceptor molecule for saccharide attachment. During KS biosynthesis, chain elongation and sulphation are coordinated events and elongation of the KS chain occurs by stepwise addition of GlcNAc or Gal coordinated with sulphation of these moieties [17, 18]. Several glycosyltransferases and sulphotransferases are involved in KS biosynthesis; these are shown in Fig. 2.2 reproduced from KEGG KS biosynthesis reference data (Map 00533) [http://www.kegg.jp/kegg-bin/show_pathway?map00533]. GlcNAc

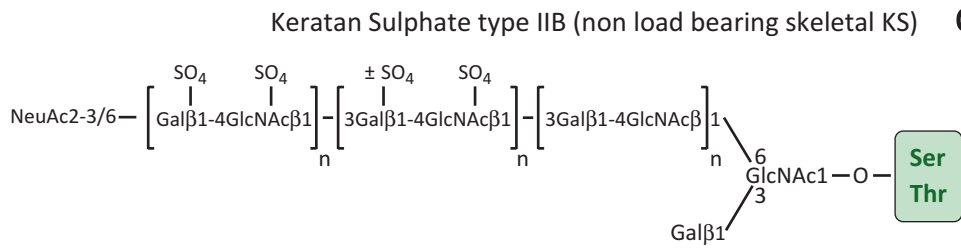
[1]Please note that the KS antibodies referred to in this chapter are directed to epitopes in the glycosaminoglycan keratan sulphate and should not be confused with anti-aminoacyl-tRNA synthetase (ARS) antibodies which have also been designated as KS antibodies [15] or the anti-cyclin D1/D2 antibody which is also referred to as 5-D-4 [16].



The KS repeat disaccharide







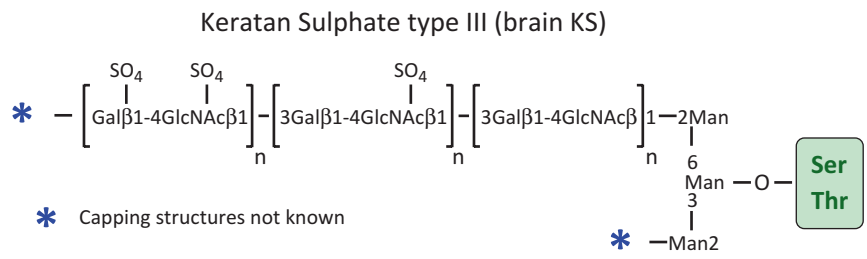


Fig. 2.1 The structural heterogeneity of KS assembled from the repeat disaccharide D-Gal-GlcNAc-6-sulphate showing pertinent features of corneal KS-I and its di-, mono-, non-sulphated and linkage regions plus fucose and sialic acid end-capping structures (**a**) and of equivalent regions in skeletal KS-II isolated from weight-bearing connective tissue (**b**) and KS-II from non-weight-bearing connective tissue (**c**) and brain KS-III (**d**)

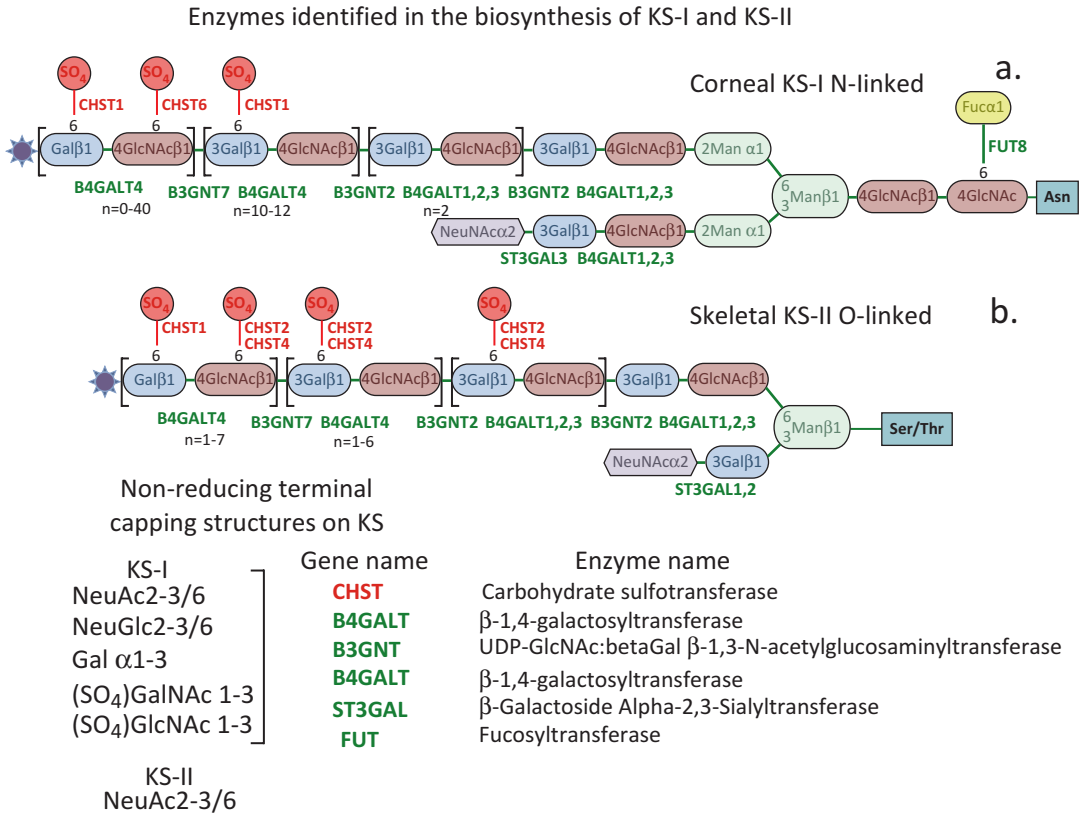


Fig. 2.2 This figure is reproduced from the KEGG KS biosynthesis reference data map (Map 00533) [http://www.kegg.jp/kegg-bin/show_pathway?map00533] which shows the major known KS biosynthetic enzymes

6-O-sulphotransferase acts only on terminal non-reducing terminal GlcNAc residues on the nascent KS chain.

Failure to add sulphate to a terminal GlcNAc residue may result in a disaccharide unit devoid of sulphate or having one sulphate group located on the GlcNAc residue only; D-Gal sulphotransferase only acts on a KS disaccharide if the GlcNAc is first sulphated giving rise to a disulphated disaccharide. Thus heterogeneous distributions of mono- or disulphation or non-sulphation can also occur along a given KS chain. GlcNAc normally undergoes sulphation more frequently than Gal in the KS disaccharide. Like all GAGs, the sulphation status of KS defines its functional properties.

2.2.1 Keratan Sulphate Biodiversity

KS has been categorized into three types on the basis of differences in the structures of the linkage region they utilize to attach to PG core proteins and in their internal structural organization (Fig. 2.1). KS-I was the first form of KS identified, cornea is the richest tissue source of this GAG, leading to its historical naming as corneal KS [19]; however, this form of KS also decorates a number of PGs with a widespread tissue distribution in a range of tissues other than the cornea thus its naming is a historical misnomer. KS-II or skeletal KS exclusively decorates the major cartilage PG aggrecan. A further form of KS has been identified in brain (KS-III) which is rare in non-

neural tissues but occurs in ~30% of all brain glycoproteins and PGs (Fig. 2.1).

Corneal KS (KS-I) is attached to Asn in PG core proteins via a complex-type *N*-linked branched oligosaccharide, whereas in cartilage, KS-II is *O*-linked via GlcNAc to Ser or Thr residues via a mucin core-2 structure [17]. Brain KS-III uses a third type of linkage to protein via mannose *O*-linked to Ser or threonine [20]. These linkage oligosaccharides are shown in Fig. 2.1. KS is a heterogeneous GAG and exhibits both variation in chain length and in sulphation along the KS chain. Five regions can be identified in KS-I: (i) the non-reducing terminal end-capped region, (ii) disulphated region, (iii) monosulphated region, (iv) non-sulphated lactosamine region and (v) the linkage region to PG core protein. Equivalent regions in KS-II and KS-III also occur but the lengths of individual regions and sulphation patterns may differ leading to a considerable level of size and charge heterogeneity in KS. Furthermore, the size distribution and degree of sulphation of KS chains increases with tissue development and maturation and the age of the connective tissues and its pathological status. High-charge-density KS has been observed associated with a number of tumours thus its analysis can be of diagnostic value.

In porcine corneal KS, the C-6 branch of the linkage oligosaccharide is extended but the C-3 branch is terminated by a single lactosamine capped by sialic acid [21]. Sulphation in porcine corneal KS is distributed non-randomly; two non-sulphated lactosamine disaccharides are present nearest to the reducing terminus but 10–12 sulphated GlcNAc disaccharides are found on the more distal part of the chain. The non-reducing terminal region is of variable length and contains disulphated GlcNAc and Gal disaccharides sulphated at C6 [22–39]. Corneal KS displays a single branch in the linker oligosaccharide; an extension of the other branch in the biantennary oligosaccharide is also occasionally possible [reviewed in [18]]. The non-reducing ends of KS-I chains are terminated with neuraminic acid, βGalNAc or αGal end-capping structures [39, 40]. Despite its name, KS-I is found in tissues other than the cornea such as in cartilage N-linked KS chains that occur on fibromodulin, lumican, PRELP (prolargin), keratocan and osteoadherin [22, 25, 38]. Aggrecan contains 2–3 *N*-linked KS chains in addition to 20 or more *O*-linked KS-II chains in the KS-rich region adjacent to CS substituted regions on the aggrecan core protein [24]. A few KS chains are also interspersed in the CS-1 and CS-2 regions in aggrecan as these differ from the KS chains of the KS-rich region in that they can be heavily modified by fucosylation and sialylation, making them immunologically distinguishable. The amino terminal G1 and G2 globular domains of aggrecan and the interglobular domain (IGD) between these contain a few small KS chains; however, these are of low sulphation and can be *N*- or *O*-linked. Some of these KS chains in G1 obscure T-cell epitopes which otherwise make the G1 domain a potent arthritogen in inflammatory arthritis. KS chains within the IGD potentiate the action of ADAMTS-4 and ADAMTS-5 which cleave in the IGD and elsewhere in the aggrecan core protein. These enzymes are important for aggrecan turnover; however, excessive ADAMTS activity results in cartilage degeneration and pathological tissue changes in OA and RA. PZP3 zona pellucida glycoprotein carries KS-I chains; however, these differ from the KS-I chains found in cornea [36]. Similarly, KS-I in fibromodulin is relatively short (8–9 disaccharides), more highly sulphated [34] and lacks the characteristic domain structure of corneal KS and its non-reducing terminal end-capping saccharides resemble those found in cartilage KS-II rather than corneal KS-I [34], thus such capping structures are tissue-specific rather than KS type-specific. KS-II in the KS-rich region of aggrecan contains 5–11 highly sulphated disaccharides, interrupted only occasionally by monosulphated KS and its non-reducing terminal region is capped by neuraminic acid attached at C3 or C6 to terminal GlcNAc. Furthermore, fucose is attached to C3 of sulphated GlcNAc throughout the KS chain but not within four residues of its non-reducing terminus [26]. KS-II from non-weight-bearing tracheal cartilage is not fucosylated, and carries only (2→3) linked neuraminic acids at the non-reducing terminus [27, 35].

Table 2.1 Antibodies developed to KS illustrate its structural complexity

Antibody	Epitope identified	Ref
TRA-1-60	Epitope sensitive to neuraminidase, keratanase-I/II and endo-β-D-galactosidase. Epitope identified Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc; this oligosaccharide is expressed on podocalyxin on pluripotent embryonic stem cells	[41–45]
TRA-1-81	Epitope resistant to neuraminidase but sensitive to endo-β-D-galactosidase, keratanase-I/II. Epitope is terminal Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc; these oligosaccharides are expressed on cell surface podocalyxin on pluripotent embryonic stem cells	[41–45]
R-10G	Low sulphation poly *N*-acetyllactosamine KS epitope	[46–49]
SSEA-1	Cell surface glycan of murine embryonic pluripotent stem cells, epitope expressed on proteoglycan and glycoprotein core proteins and bioactive lipids	[50]
"i" antigen	Human autoantibody to a non-branched epitope in non-sulphated poly-*N*-acetyllactosamine	[51–55]
"I" antigen	Human autoantibody to a branched epitope in non-sulphated poly-*N*-acetyllactosamine regions of KS	[51–55]
4C4	Highly sulphated KS on embryonic tumour cell podocalyxin	[56]
5D4	Hexa-sulphated KS saccharide	[57, 58]
MZ15	Hepta and octa-saccharide KS oligosaccharides	[58, 59]
1B4	Tetrasulphated hexasaccharide in linear KS	[58]
3D12/H7	Trisulphated fucosylated poly-*N*-acetyllactosamine KS chains located in the CS-1 and -2 region of aggrecan core protein	[60]
D9B1	A sialo-KS epitope on endometrial KS-PGs	[61–63]
6D2/B5	Fucosyl-KS epitope	[64]
SV2	High sulphation KS chains on SV2 PG	[65, 66]
EFG-11	Tri KS disaccharides	[67]
1/14/16H9	Specific equine KS antibody	[68, 69]
BKS-1(+)	D-GlcNAc 6-sulphate KS stub neo-epitope exposed by keratanase-I/II, endo β-D-galactosidase digestion	[70]

Abbreviations: *TRA* trafalgar antigen/tumour rejection antigen, *SSEA* stage-specific embryonic antigen. These antibodies identify non-sulphated epitopes in poly-lactosamine regions occurring in KS

2.2.2 Keratan Sulphate Antibodies

Monoclonal antibodies to KS (Table 2.1) react with extracts from most mammalian tissues, at least 16 ECM PGs substituted with KS and several intracellular and cell-associated KS-PGs have been identified [reviewed in [17, 18]]. All GAGs other than KS contain at least one negative charge per disaccharide, the lack of uronic acid in KS and variable sulphation of its lactosamine residues results in charge heterogeneity in KS [17, 18]. Furthermore, a number of poly-*N*-acetyl lactosamine-modified proteins exist, which would be classified as KS-PGs if some of their residues were sulphated [32]. The development of MAb R10G and 1B4 allows KS-PG species of low sulphation and mucin-like proteins containing lactosamine regions which contain GlcNAc and Gal residues that are sulphated to be identified as KS-PGs (Fig. 2.3). Formerly, antibodies such as 5D4 and MZ-15 which detect high-charge-density KS glycoforms were routinely used in this research area; however, these do not detect such low sulphation forms of KS, thus a new aspect of the biology of KS-PGs of low sulphation is now emerging [59, 71, 72].

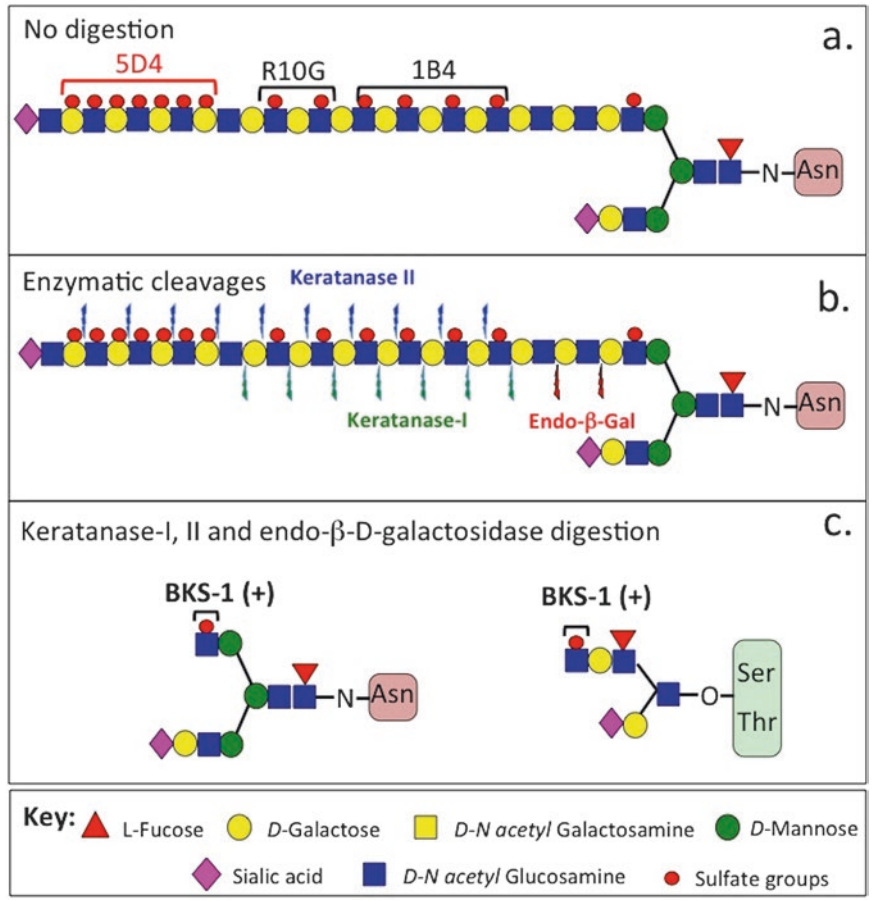


Fig. 2.3 Putative antibody recognition sites on native undigested KS-I (**a**) and keratanase-I, keratanase-II and endo-β-D-galactosidase cleavage sites on the KS chain (**b**) which generate the neo-epitope BKS-1 (+) stub KS epitope

2.2.3 Keratan Sulphate Complexity in Healthy and Diseased Tissues

KS and Its Specific Roles in Tumours, Spinal Cord and Brain

Analysis of GAGs associated with normal and tumour tissues and tumour cells [13, 73–83] and their secretions [77, 84] has identified the glycan signatures of pathologic tumourogenic tissues and shown these are of diagnostic and prognostic value [13, 85]. Changes in the PG compositions associated with tumour masses have also been identified [86, 87]. KS is a prominent component of many tumours including carcinomas of the genital tract [85], prostatic secretory cells [73], brain and ovarian tumours [82], papillary carcinomas of the human thyroid gland [88] and granular cell tumours [74]. The human embryonal carcinoma marker antigen TRA-1-60 identifies a sialylated KS-PG [41]. Chondrosarcoma cells synthesize a characteristic KS-PG in long-term culture [86]. Improved methodologies have been developed for the structural characterization of KS produced by ovarian and brain tumours [82]. KS-substituted isoforms of thyroglobulin and transferrin are uniquely elaborated in papillary thyroid carcinomas [46]. Highly sulphated KS is synthesized in malignant astrocytic tumours [76, 89], and glioblastoma [75]. Lumican is a prominent KS-PG associated with a number of tumours (Table 2.2) and has roles in the regulation of tumour cell growth, migration and attachment to ECM components [110, 111, 121, 122]. Another

Table 2.2 Lumican influences many different tumour types

Tumour type	Features affected by lumican	Ref
Melanoma A375, B16F1 cells	Inhibition of MMP-14 and tumour cell attachment and proliferation	[90–96]
Pancreatic cancer	Inhibition of tumour cell growth [97]. Lumican is expressed in alpha cells of pancreatic islets and pancreatic cancer cells [98]. Lumican stimulates growth and inhibits replication and invasion of human pancreatic cancer cells [99, 100] and pancreatic ductal adenocarcinoma [101]	[97–101]
Giant cell bone tumour	Down-regulation of lumican may serve as a biomarker of metastatic and recurrent giant cell bone tumours	[102]
Prostate cancer	Anti-tumour activity. Inhibition of the migration and invasion of lymph node, bone and brain metastatic prostate cancer cells	[103]
Colon carcinoma Colorectal cancer	Overexpression of lumican upregulates gelsolin and filamentous actin reorganization [104] and is associated with good outcome in Stage II, III Colon carcinoma [105]. Lumican expression in advanced colorectal cancer with nodal metastasis correlates with poor prognosis	[104, 105] [106, 107]
Osteocarcinoma	Regulates tumour cell adhesion by modulating TGFβ2 activity [108]. Lumican expression is positively correlated with the differentiation and negatively with the growth of human osteosarcoma [109]	[110, 111]
Breast cancer	Reduced expression of lumican is associated with poor outcome in node-negative invasive breast cancer. Lumican influences ECM organization	[112, 113]
Adenocarcinoma and squamous cell carcinoma of lung	Upregulation of lumican inhibits tumour cell migration and cellular proliferation	[114, 115],
Carcinoid tumours, neuroendocrine cell carcinoma	Cytoplasmic lumican in neuroendocrine tumour cells is associated with the RER, cellular granules and the interspaces of stromal collagen fibres. Higher cytoplasmic expression of lumican in carcinoid tumours compared to neuroendocrine carcinomas may slow the growth of the former tumour cells	[116]
Salivary pleomorphic adenomas	Lumican expression is associated with the formation of mesenchyme-like elements in salivary pleomorphic adenomas	[117]
Uterine cervical cancer	Lumican protein accumulates in uterine cervical cancer cells at the periphery of cancer nests	[118]
Endometrial cancer	Endometrial cancer is the most common form of malignant gynaecological tumour. Lumican is strongly associated with these tumours; however, it's functions in such tumours still has to be determined	[119, 120]

KS-PG, podocalyxin, has also been found associated with malignant astrocytic tumours [89]. Monoclonal antibody 4C4 specifically recognizes KS-PG on human embryonal carcinoma cells [56]. KS has been identified as a prominent component of pathological brain tissues. KS is produced by microglial cells in the development of amyotrophic lateral sclerosis (ALS) [123–126]. A reduction in KS levels in brain tissues accelerates the development of ALS [127] and Alzheimer's disease (AD) [128, 129].

In the intact normal spinal cord, microglial cells and macrophages express the 5D4 KS epitope; however astrocytes do not [130]. A focal upregulation of 5D4 reactivity occurs associated with glial scar formation following spinal cord

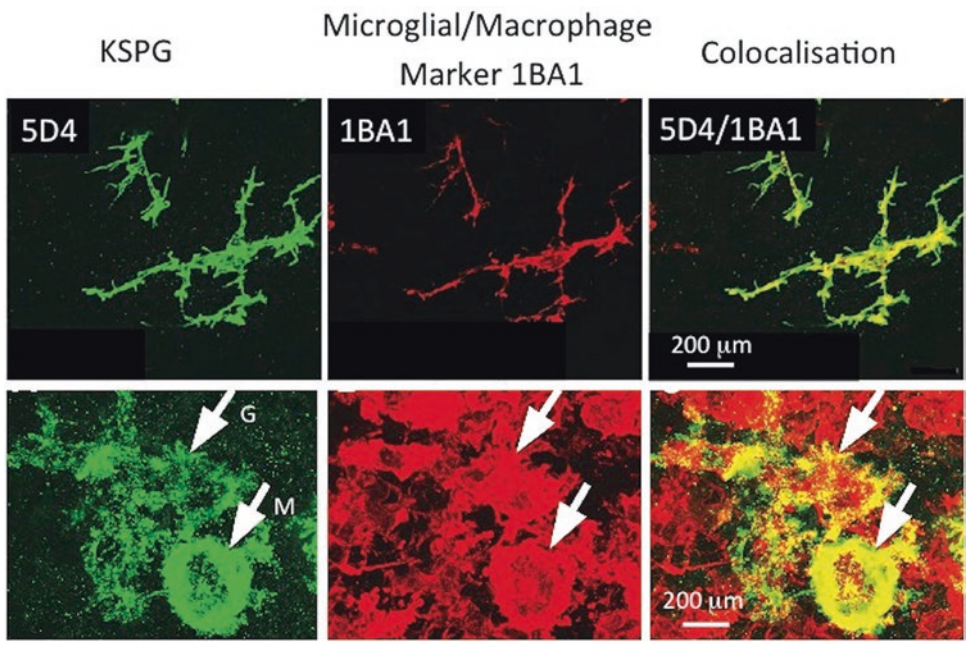


Fig. 2.4 Immunolocalization of the 5D4-positive KS epitope synthesized by microglial cells and macrophages in rat spinal cord follow spinal cord injury. The arrows indicate glial cells (G) and macrophages (M) which synthesize 5-D-4 KS. Areas of co-localization are indicated in yellow. (Modified from Lindahl et al. [128] with permission under the auspices of Creative Commons Attribution 4.0 International License (CC-BY))

injury apparently due to glial cell activation and an influx of macrophages to the lesion site (Fig. 2.4). Proteoglycans are upregulated in the spinal cord lesion site and this stabilizes this structure; however, the KS and CS side chains of these PGs strongly inhibit nerve outgrowth and axonal regeneration [131–133]. Therapeutic administration of keratanase, chondroitinase B and chondroitinase ABC significantly improves spinal cord regeneration in experimental rat models and suggesting these as appropriate therapeutic interventions to improve recovery of human spinal cord injury [131, 134, 135]. Fragmentation of aggrecan occurs in the normal intact spinal cord through the action of aggrecanase and metalloprotease enzymatic activity and the abundance of aggrecan fragments increases with spinal cord injury [136–141]. Up-regulation of ADAMTS-4, 5 in the spinal cord lesion site is associated with areas of improved repair post injury and these have been suggested to be of therapeutic value, however these findings need to be carefully evaluated [139, 142].

KS also has roles in the pathogenesis of ALS and in the activation and proliferation of microglial cells [124]. KS binds to Shh and regulates the differential switch from motor neuron to oligodendrocyte during spinal cord development [143]. Phosphacan containing high-charge-density 5D4-positive KS chains regulates the development of the mouse visual cortex [144]. KS inhibits neural regrowth [145] and directs the development of the trigeminal nerve during corneal development [146]. KS has interactive properties with a large number of nerve regulatory proteins through which it can regulate neural development through interaction with members of the Robo, Slit, Ephrin, Ephrin receptor and Semaphorin families and two further nerve growth factor receptors [147]

KS Is a Component of Cell Surface Glycans Expressed by Human Stem Cells.

Wu et al. 2019 [148] applied state-of-the-art glycan array technologies to compare the glycans bound by five antibodies that recognize

carbohydrate cell surface epitopes on human stem cells. A panel of sequence defined glycans confirmed that the common epitope identified by these antibodies was the non-reducing terminal disaccharide Galβ-4GlcNAc contained within the common epitope Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAc (Table 2.3). The interactive partners for specific stem cell surface glycan sequences in the niche environment are important in the determination of events in stem cell differentiation. It is important to define the precise epitope identified by antibodies which have been used to identify human stem cells.

Weyers et al. 2013 [150] previously reported that corneal KS bound FGF-2 and Sonic hedgehog (Shh). Wu et al 2019 [148] conducted glycan microarray analyses on FGF-1, FGF-2, Shh, int/Wingless (Wnt)-3a, BMP-2 and BMP-4 and reported positive binding of FGF-2 and BMP-2. The sulphation density was an important determinant for FGF-2 and BMP-2 binding, Keratanase II generated 6 mers displayed positive binding with KS oligosaccharides containing 4, 5 or 6 sulphate groups producing positive binding with FGF-2 and BMP-2. KS oligosaccharides bearing less than 4 sulphate groups did not bind FGF-2 or BMP-2 [148] but were bound by MAb R-10G [47, 149, 150]. In the initial studies on the R-10G determinant expressed on podocalyxin, this was suggested to be an O-glycan devoid of substitution with sialic acid and fucose [47]. Evidence of sulphate as a component of the R-10G epitope was obtained after keratanase II digestion and identification of the binding disaccharide Gal-GlcNAc(6S) [47]; Gal-GlcNAc(6S)-Gal-GlcNAc(6S) was identified as a R-10G epitope in a following study [149], the R-10G-binding glycan epitope [148] thus falls in the 4–6 mer size range.

Natural Killer Cells Express Cell Surface KS-Proteoglycans with Effector Functions

Natural killer (NK) cells are CD3 $^{-}$, CD16+, CD56+ large granular lymphocytes which recognize and eliminate virus-infected, malignant and antibody-coated target cells in the innate immune response [151, 152]. Two functionally distinct populations of peripheral blood NK cells have a differing surface expression of an isoform of the neural cell adhesion molecule CD56. CD56 bright NK cells are an undifferentiated cell type which proliferate in response to exogenous cytokines, but have poor cytolytic activity [152]. These contrast with the CD56dim NK cell population which is a more differentiated cell type, poorly responsive to exogenous cytokines, but display potent cytolytic activity [152]. The critical functioning of the NK cells of the innate immune system is critically dependent on NK cell trafficking and homing. Differentiation of NK cells is accompanied by the expression of a cell surface mucin-like glycoprotein bearing an NK cell-restricted KS-like lactosamine PEN5 epitope, a post-translational modification of the platelet selectin glycoprotein ligand-1 (PSGL-1) [151]. This PEN5 epit-

Table 2.3 KS disaccharide and oligosaccharides identified by MAb R-10G

Structure	Reference
Galβ-4GlcNAc 6S	[47] [149] [148]
Galβ-4GlcNAcβ-3Galβ-4GlcNAc 6S 6S	
Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAc 6S 6S 6S	

ope on PSGL-1 is a unique binding site for L-selectin with binding properties independent of tyrosine sulphate epitopes on PSGL-1. PSGL-1 binds to P-, E- and L-selectin expressed by platelets, endothelial cells and leukocytes respectively. The PEN5, sulphated KS-lactosamine epitope on PGSL-1 is selectively expressed on mature CD56dimCD161 NK cells, conferring an ability to bind to L-selectin as a functional NK-homing-trafficking receptor delivery system in the innate immune response. Biochemical and immunochemical studies show that PEN5 is an unusual sulphated poly-N-lactosamine KS epitope [151]. A monoclonal antibody raised to PEN5 (5H10) immunoprecipitated two polydisperse membrane-bound glycoproteins, PEN5α (120–170 kDa) and PEN5β (210–245 kDa) from NK cells. Enzymatic deglycosylation using keratanase-I, *O*-glycanase and PNgase, reduced the apparent molecular weights of these proteoglycans in SDS-PAGE by 80–90%, confirming PEN5 as a KS mucin-like epitope [152]. Chondroitinase ABC, heparitinase and heparanase had no effect on the molecular weight of PEN5α oρ PEN5β and neuraminidase produced a moderate reduction in size indicating a small level of terminal sialic acid substitution on this KS epitope. However, the failure of distinct KS antibodies such as 1B4, 2D3, 3D2, 4D1, 8C2 and 5D4 to label NK cells indicated that the form of KS identified by the PEN5 5H10 Mab was restricted to NK cells and was not widely distributed in KS from other sources. Further experiments showed PEN5 did not occur widely in other KS proteoglycans in cartilaginous tissues [152].

The cell surface expression of the PEN5 epitope is downregulated by stimuli that induce NK cell proliferation, and is absent in leukemic NK cells thus PEN5 is a developmentally regulated poly-N-lactosamine KS epitope expressed by non-proliferative NK cells fully primed to cytolytic effector functions [151]. Furthermore, this PEN5 epitope is insensitive to digestion with trypsin and chymotrypsin in these NK cells and may exert a cyto-protective effect on NK cells involved in cytolytic events [152].

2.2.3.1 Mucin-Type Glycoproteins

The Role of KS Substitution in Tumour Development

Membrane-bound and secreted mucin-type glycoproteins contain GalNAc, GlcNAc, Gal, Fuc, N-acetyl neuraminic acid attached to their core proteins through *O*-linkage to Ser and Thr residues on their tandem repeat domains leading to a bottlebrush-type structure reminiscent of PGs such as aggrecan (Table 2.4). A family of sulphotransferases can sulphate the GlcNAc and Gal residues in mucins, and thus some mucins carry KS chains [163], MUC1, MUC4 and MUC16 synthesized by normal cultured bronchial epithelial cells bear 5D4-positive KS [164]. MUC16 is the largest transmembrane mucin with a molecular weight ranging from 2.5 to 5 MDa. MUC16 lubricates and protects the mucosal epithelium of the upper respiratory tract, ocular surface, mesothelial pleural, peritoneal and lining tissues of the male and female reproductive organs. MUC16 contains extracellular and transmembrane domains as well as a cytoplasmic domain which interacts with the ERM cytoskeletal actin-binding proteins ezrin, radixin and moesin. MUC16 is also associated with tumour cells, and its extracellular domain is cleaved from ovarian cancer cell surfaces into the bloodstream where it is useful as a tumour biomarker through the identification of a peptide epitope (CA125) which also promotes cancer cell proliferation [71, 72, 165]. Cultured human tracheobronchial epithelial cells synthesize 5D4 KS-positive MUC1, MUC4 and MUC16 tethered to cilia and microcilia; however, no PGs have been detected in the epithelial glycocalyx (Fig. 2.5). KS on the mucin-associated cilia and ciliary plumes provide a protective layer extending as far as 100 μm from the epithelial cell surface [164].

The sulphated glycans on epithelial mucins effect cell adhesion and regulate the biosynthesis, half-life and biological roles of glycoproteins controlling lymphocyte homing and inflammation in the epithelial mucosa. Two sulphotransferase families transfer sulphate from 3-phosphoadenosine 5-phosphosulphate (PAPS) to C3 of Gal (Gal3ST) or C6 of GlcNAc

Table 2.4 *O*-glycan core mucin-type acceptor structures sulphated on Gal or GlcNAc

O-glycan acceptor	Mucin source	Sulphation position	Reference
Core 1 Galβ1-3GalNAc-	Rat gastric and salivary mucins	C6 on extending GlcNAc	[153]
Core 2 GlcNAcβ1-6Galβ1-3	Rat mammary adenocarcinoma	C6 on GlcNAc	[154–156]
Acceptor structure unspecified	Pig gastric mucin	C6 on GlcNAc	[157]
Core 3 GlcNAcβ1-3GalNAc- repeat NAcetyl lactosamine disaccharides	Pig zona pellucida glycoproteins	C6 on GlcNAc in *O*- and *N*- linked glycans	[158]
Specific acceptor structure not specified	Cystic fibrosis respiratory mucins	C3 on Gal and C6 on GlcNAc on multiple complex *O*-glycans	[159–161]
Core 6 GlcNAc β1-6GalNAc-	Rat bone sialoprotein	C6 on GlcNAc	[162]

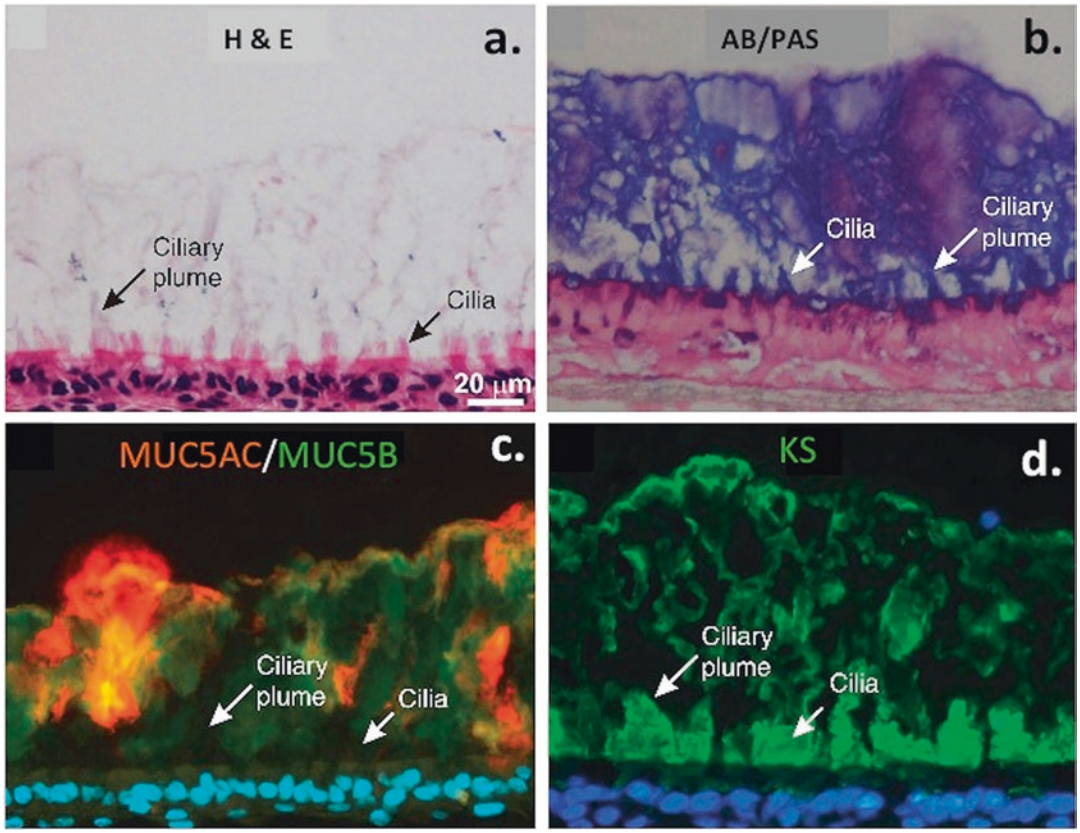


Fig. 2.5 KS localized in mucus and mucins of the mucosal surface of human tracheobronchial epithelial cell cultures visualized using haematoxylin and eosin (**a**), Alcian blue-periodic acid–Schiff staining (**b**) or by immunolocalization of MUC5AC, MUC5B (**c**) and KS (MAb 5D4) (**d**) using specific antibodies. Panels **c** and **d** were counterstained with DAPI to visualize cell nuclei. Note the height of the accumulated mucus layer ~100 µm, the intense staining of KS in the periciliary layer and plumes of material extending from the ciliary tips into the mucus ciliary plumes (**d**) while excluding the polymeric mucins in panel (**c**). Intracellular mucins are not apparent in these images since their fluorescence intensities did not reach the detection threshold appropriate for use in the visualization of the strong extracellular immunolocalizations. Scale bar 20 µm. (Figure reproduced from Kesimer et al. [164] with permission, Springer Nature, Mucosal Immunology (license number 4605370414328))

(GlcNAc6ST) in mucins. The ubiquitous mucin core 1Gal3ST acts on O-linked Galβ1–3GalNAc α-R in most tissues, with high activity levels in rat colonic mucosa and is also upregulated in inflamed cartilage, intestine and lung tissues in tumour development. KS has been immunolocalized to the cell-associated mucins MUC1, MUC4 and MUC16 [164]. O-glycan mucin core structures 1–4 and 6 act as potential substrates for sulphotransferases [158, 162, 166–168], sulphation on Gal and GlcNAc residues of N-acetyl lactosamine occurs at C3 of Gal and C6 of GlcNAc [163]. Human mammary epithelial cells synthesize PGs containing *O*-linked sulphated GlcNAc attached to core 2 structures [169] (Table 2.4). MUC-1 in human endometrial tissue carries 5D4-positive KS and a sialo-KS epitope recognized by Mab D9B1 [61]. These epitopes convey adhesive and anti-adhesive properties which regulate embryo implantation [61]. These KS epitopes are independently regulated in the endometrial endothelium due to hormonal control with the 5D4 epitope abundant on the luminal epithelial surface until implantation, thereafter it gradually disappears, D9B1-binding sites are retained in the luminal endometrial epithelium following implantation [170].

An endothelial mucin-like adhesion molecule (Glycam-1) binds L-Selectin through C6 sulphated GlcNAc and Gal on O-linked Sialyl Lewis^X like structures [171, 172]. Sulphation of Sialyl Lewis^X structures significantly improves their L-Selectin binding properties. The sulphation motifs on mucins act as binding modules for bacteria but also protect the mucins from depolymerization by bacterial glycosidases. Changes in mucin sulphation alter growth factor interactions, leucocyte homing and adhesion under inflammatory conditions [173]. In monocytes, TNFα induces expression of 6-sulpho N-acetyl lactosamine (LacNAc)/Lewis X epitopes on *N*- and *O*-linked cell surface glycans altering their migratory and adhesive properties [173]. Cell surface and secreted mucins in ovarian cystadenoma [174] or human bronchial mucins [33, 175] also carry such sulphated Lewis X L-selectin ligands which promote leucocyte attachment to the endothelium [176–178]. The sulphate content of mucins is decreased in colon cancer and in ulcerative colitis [179–182] due to degradative effects on mucins by bacterial sulphatase activity [183]. A significant reduction in mucin sulphation has also been observed in colorectal adenoma cells as they progress to a cancerous state. This decrease is due to decreased core 1 Gal3ST and GlcNAc6ST expression [184]. Lower Gal3ST activity is also a feature of colon cancer [185–187] and breast cancer cells compared to normal mammary cells [166]. The mucin core structures biosynthesized and their associated modifications in cancer [187] influence the amount of mucin sulphation. Alterations in the expression patterns of sulphated mucins and sulphotransferases in inflammatory diseases and cancer alter the distribution and density of mucin sulphation motifs and adversely influence disease progression [163].

2.3 Brain Contains a Number of Multifunctional KS-PGs

A number of diverse KS substituted PGs have been identified in the brain (Fig. 2.6). Aggrecan is a component of perineuronal nets which surround and protect neurons and promote neuritogenesis and synaptic plasticity [188]. Podocalyxin is a transmembrane KS-PG with cell-signalling capability widely distributed in neurons. In embryonic tissue podocalyxin isolated from pluripotent neuroprogenitor cells contains low sulphation KS chains and has been used as an antigen for the production of antibodies which identify these low sulphation KS glycoforms. However, in pathological neural tissues, tumour cells express podocalyxin decorated with high-charge-density KS glycoforms identified by antibodies 5D4, and MZ-15 [57, 58] and these may also be of diagnostic value (Fig. 2.6).

Podocalyxin is an anti-adhesive transmembrane neural KS-polysialylated proteoglycan/glycoprotein with essential roles to play in neural development [189, 190] and is also a marker

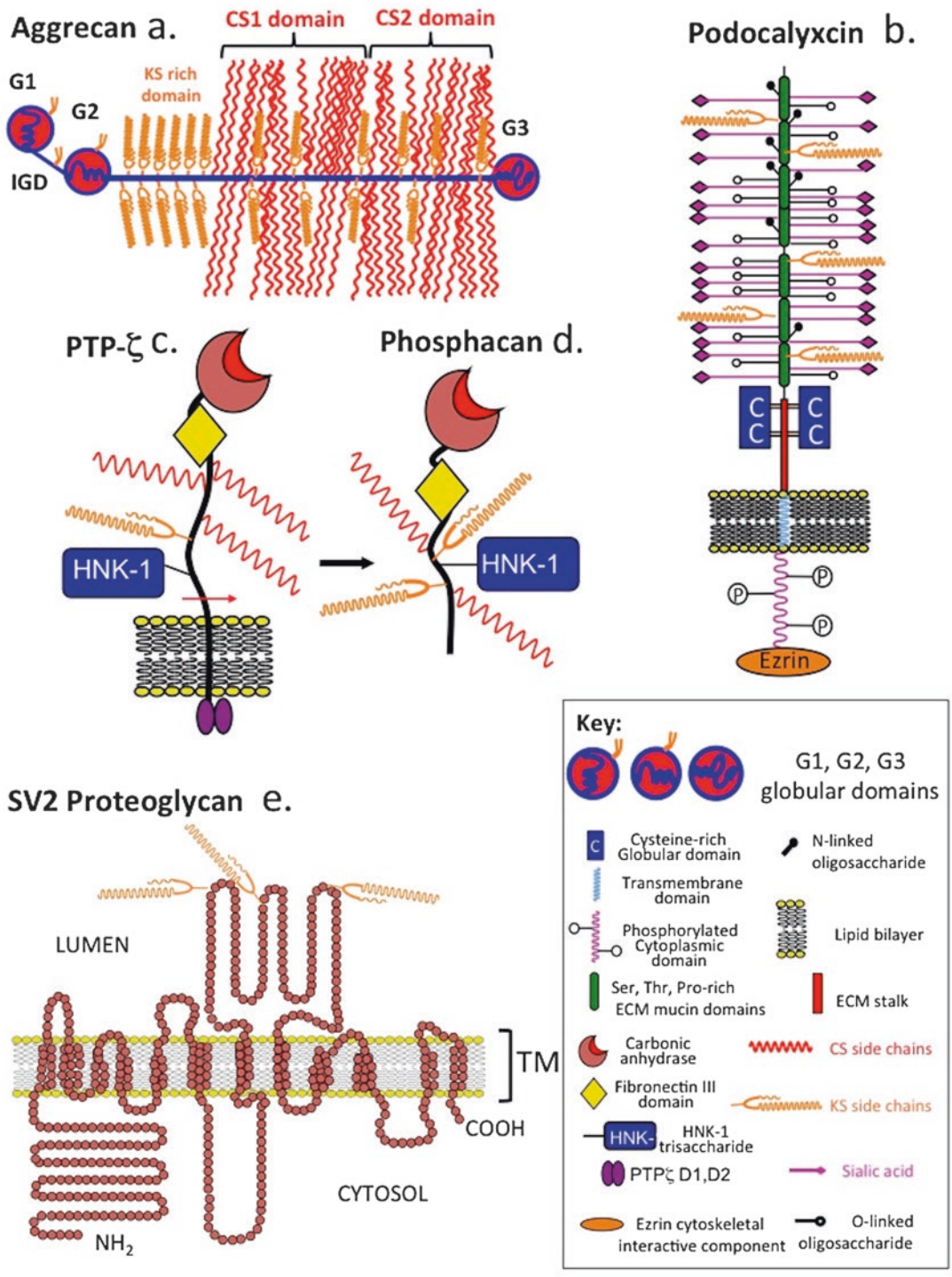


Fig. 2.6 Structural representations of the major extracellular and cellular CNS/PNS KS proteoglycans. Aggrecan (**a**), podocalyxin (**b**), RPTP-ζ (**c**), phosphacan (**d**) and SV2 proteoglycan (**e**). Note that the structure depicted in (**a**) is of human aggrecan, rat aggrecan does not have a KS-rich region. (Figure modified from Caterson and Melrose [17] with permission under the auspices of Creative Commons Attribution Non-Commercial License http://creativecommons.org/licenses/by-nc/4.0/)

of human embryonic and induced pluripotent stem cells [191]. Podocalyxin is upregulated in glioblastoma formation and in astrocytomas [75, 76, 89, 192–195], and has been developed as a prognostic factor for various cancers [196, 197]. The sulphation status of the KS chains on podocalyxin on normal embryonic cells and tumour cells differ with the former expressing a low sulphation KS detected by MAb R-10G [47, 48, 149] while tumour cells produce a high sulphation KS chain [89] detected by antibodies such as 5-D-4, MZ-15 or 4C4 [56–58] (Table 2.1).

Two cytosolic adaptor proteins, Na+/H+-Exchanger Regulatory Factor 2 (NHERF2) and Ezrin, interact with the cytoplasmic tail of podocalyxin in kidney and similar interactions with cytoskeletal components also occur in neural tissues exerting regulatory effects on cell signalling and downline effects on neural behaviour during the development and repair of the CNS/PNS [198, 199]. Neural migration and axonal guidance are governed by cues from many ECM molecules (netrins, semaphorins) which exert either attractive or repulsive cues. Podocalyxin is not essential for neural migration to occur but can modulate this process [188]. Cell–cell contact and adhesion to the ECM contribute to neural assembly processes. Adhesion molecules such as NCAM and L1 have important roles to play in axonal growth, neural migration and synapse formation. Coordination of ECM signals is essential in such developmental processes. Podocalyxin has essential roles to play in neuritogenesis and synaptogenesis [200–202]. Podocalyxin co-localizes with synapsin and synaptophysin in synapse vesicle formations [189]. Synaptophysin is a major synaptic vesicle protein which coordinates the endocytosis of synaptic vesicles during neural stimulation [203]; synapsin tethers synaptic vesicles to cytoskeletal components preventing premature vesicle release into the synaptic gap coordinating neurotransmitter release from the synaptic vesicles [204–207].

2.4 SLRPs and Their Roles in Cell Migration, Proliferation and Regulation of Growth Factors and Inflammatory Cytokines in a Diverse Range of Tissues in Health and Disease

The SLRPs have multiple functional roles in soft connective tissue ECMs where they regulate collagen fibrillogenesis and regulate growth factor and inflammatory cytokine activities (Fig. 2.7). Not only do the SLRPS maintain the integrity of tissues but their levels are elevated in OA and RA [209] and in animal models of OA [210]. Lumican binds to C1q and regulates complement activation contributing to innate immune protection [211] and may also contribute to the OA/RA pathogenic processes. Specific SLRP members such as lumican regulate cell migration and proliferation and have roles to play in tumour growth, local invasion, extravasation and invasion of remote anatomic sites [111].

Lumican plays essential roles in the regulation of collagen fibrillogenesis in different ECMs; however, there is considerable redundancy in the SLRPs. Lumican is also expressed in the developing bone matrix. Real-time PCR OF MC3T3-E1 cell cultures showed that the expression of lumican increased as the osteoblast culture differentiated, suggesting a role for lumican in the regulation of collagen fibrillogenesis in bone matrices [212]. During early embryonic murine development (E11 to E13), lumican is mainly expressed in the cartilaginous rudiments; however, by E14 to E16 lumican expression is more prominent in the developing bone. Lumican is secreted by differentiating and mature osteoblasts and can be used as a marker to distinguish proliferating pre-osteoblasts from the differentiating osteoblasts [212]. Lumican, keratocan and osteoadherin are all class II SLRPs [213] which interact with TGF-β, BMP4, WISP-1 (Wnt1-inducible secreted protein-1), von Willebrand factor, PDGF, TNF-α and IGF-I forming growth factor concentration gradients

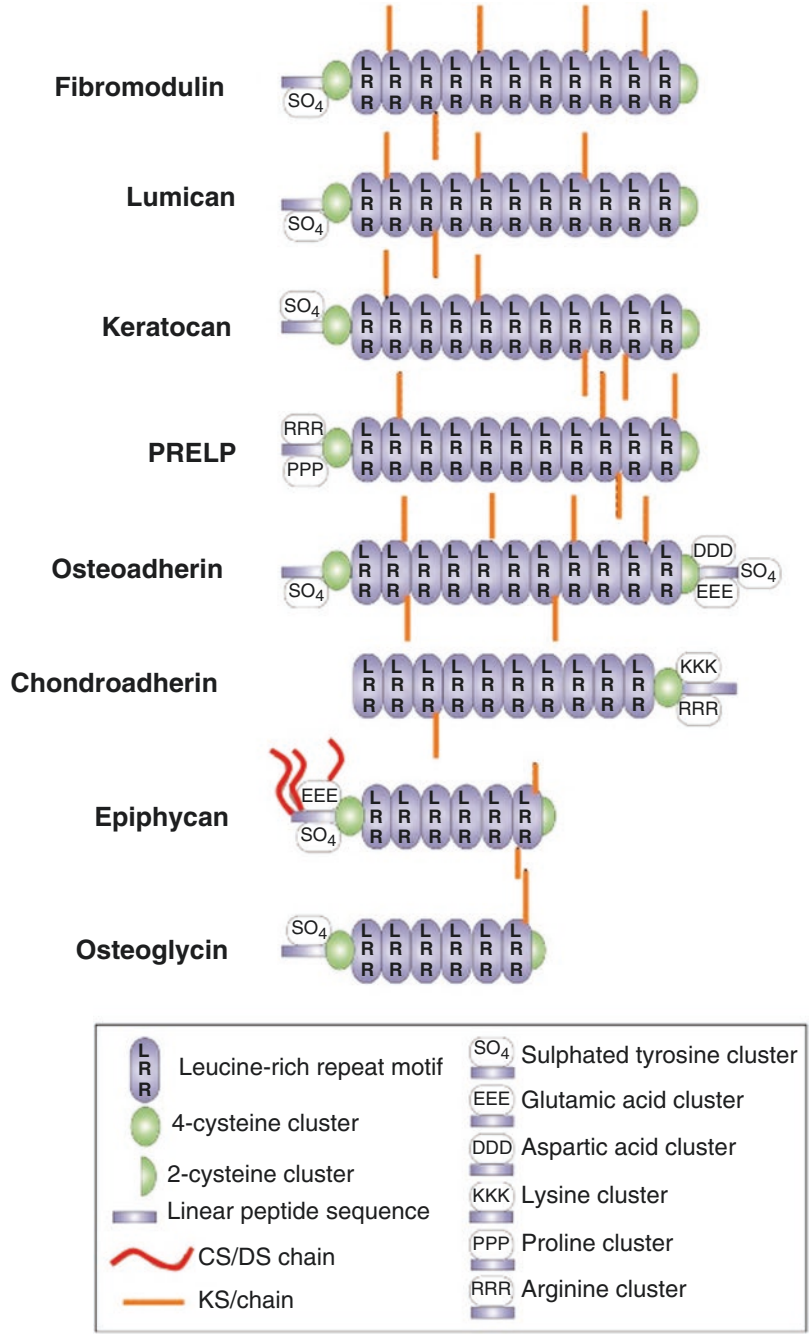


Fig. 2.7 Domain structure of KS substituted SLRP family members which are found in the CNS/PNS, and tensional and weight-bearing connective tissues figure adapted from Stanton et al. [208] with permission Elsevier, Biochim Biophys Acta (license number 4605380480747)

controlling their bioavailability to cells and pericellular interactions they participate in with cell-surface receptors, modulating cell-ECM interactions which modulate tissue development and homeostasis [213]. Osteoadherin (osteomodulin) is a 49,116-Da protein containing 11 leucine-rich repeats (LRRs), 3-4 tyrosine sulphate residues at the N-terminus, and six potential glycosylation sites for N-linked KS chains within the LRR region. Osteoadherin shows 42% sequence homology to keratocan and 37–38% identity to fibromodulin, lumican and

PRELP [38]. Osteoadherin promotes $\alpha_V\beta_3$-integrin-mediated cell binding. Osteoadherin has been isolated as a minor leucine- and aspartic acid-rich KS-PG found in the mineralized matrix of bone [214]. Osteoadherin is a relatively acidic protein which binds to hydroxyapatite and to osteoblasts through $\alpha_v\beta_3$- integrin and has been immunolocalized to pre-dentin during tooth formation [215] (Fig. 2.8).

2.5 Lumican-Specific Roles in the Regulation of Tumour Development

Lumican is a class II SLRP which bears significant levels of homology with other class II SLRPs such as keratocan, fibromodulin, lumican and PRELP. Lumican is the only SLRP which occurs with such a high frequency in tumourogenic tissues leading to the proposal of lumican as a tumour cell marker.

SLRPs organize the cartilaginous and many other soft connective tissue ECMs where they have functional roles to play in tissue development, remodelling and in pathological changes in these tissues [209]. OA is a progressive degenerative condition affecting the articular cartilage, meniscus, synovium, subchondral bone and infrapatellar fat pad in the knee joint [216, 217]. With the development of OA, PGs in these tissues undergo proteolytic degradation and some of the fragments so generated have been suggested as potential biomarkers of this disease process. Characteristic fragmented forms of the CS/DS-substituted PGs aggrecan, decorin and biglycan also occur in OA. Fibromodulin and lumican are structurally homologous sharing 47% identity in their primary structures and both can have 4 small N-linked KS chains [218, 219]. Like all class II SLRPs, fibromodulin and lumican contain 11 LRRs which facilitate their interactions with other ECM components including type I and type II collagen which regulates fibril spacing and the fibrillogenesis process. Lumican regulates the regularly orthogonally spaced fine collagen fibrillar arrangements in the cornea essential for optical clarity [220–226]. Fibromodulin is more prominent in the limbus and sclera where it stabilizes large collagen fibre assembly which mechanically support the eyeball [222, 227, 228]. Fibromodulin has N-linked KS attachment sites on Asn residues at positions 127, 166, 201, 291 and 341 in the core protein although only four of these sites are occupied by KS at any one time. Lumican also contains four N-linked KS chains located within the central LRR region at Asn 88, 127, 160 and 252. In addition, both of these SLRPs contain N-terminal sulphated tyrosine clusters, with fibromodulin containing up to nine of these residues and lumican two [211, 229], this localization of charge facilitates interactions with growth factors in a similar manner to HS interactions with growth factors.

Despite this similarity in structural form, ADAMTS-4, ADAMTS-5 [208], MMP-2, -3, -13 and -14 variably degrade fibromodulin and lumican during the etiopathogenesis of OA [230], releasing intact or fragmented forms of fibromodulin or lumican from articular cartilage, meniscus and other joint tissues. These SLRP fragments act as DAMPs activating TLR-2 and -4 initiating innate inflammation, and pain pathways [90, 231]. Lumican also augments LPS signalling through cell surface CD14, a bacterial lipopolysaccharide co-receptor which interacts with TLRs leading to NFκB activation, cytokine secretion and an inflammatory response [90]. As already noted despite similarities in structure, fibromodulin and lumican display differential susceptibilities to degradation by MMPs and ADAMTS-4 and -5. Thus while fibromodulin is susceptible to degradation, lumican is far less susceptible. This may be due to lumicans ability to act as an MMP inhibitor [232]. Lumican binds to and completely inactivates MMP-14 activity in B16F1 melanoma cells [232], inhibiting cell migration, angiogenesis and cell–ECM interactions that normally promote tumour progression [91, 233]. Lumican contains an MMP inhibitory peptide module in LRR-9 named Lumcorin [90]. MT1-MMP cleaves lumican abrogating this suppressive activity in tumour cells [234].

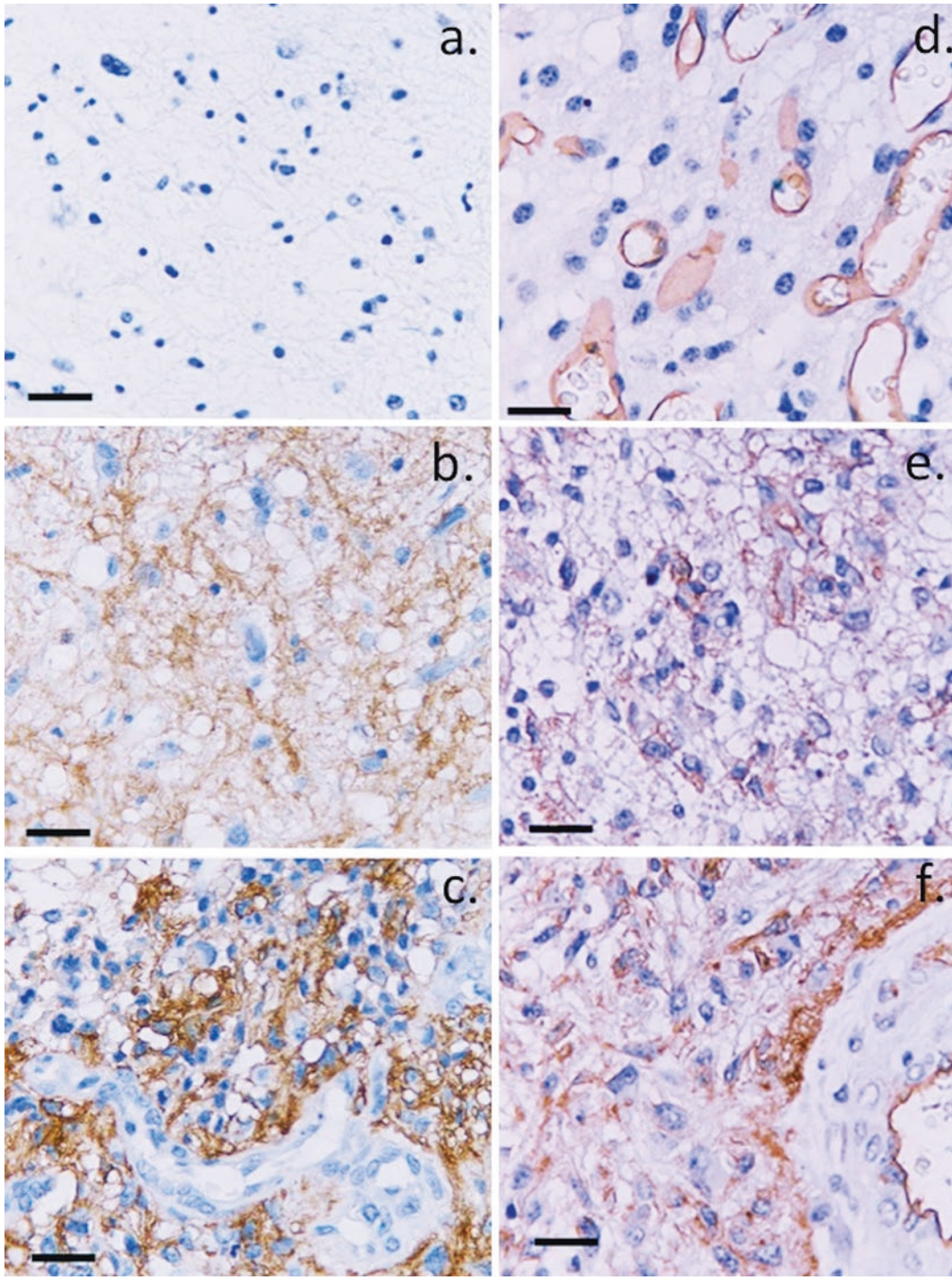


Fig. 2.8 Upregulation of podocalyxin expression in astrocytoma in the brain. Normal brain tissue showing an absence of detectable podocalyxin (**a**). Assorted views of astrocytomas and immunolocalization of podocalyxin (**b**–**f**). (Images (**a**–**c**) modified from Kato et al. [76] with permission Elsevier, Biochemical and Biophysical Research Communications (license number 4605390803553). Images (**d**–**f**) modified from Hayatsu et al. [89] with permission Elsevier, Biochemical and Biophysical Research Communications (license number 4605390045819))

2.6 SLRPs and Cancer

Specific Roles of Lumican in Tumour Cell Regulation

The tumour microenvironment decisively controls cancer development by establishing a complex interplay between cancer cells and their surrounding stromal components which directs disease progression [235]. The tumour stroma is composed of collagens, PGs, structural glycoproteins and cell adhesive proteins. Lumican prevents invasion of the ECM by tumour cells through intrinsic mechanisms which downregulate cell signalling processes that would otherwise promote cancer cell proliferation [236]. SLRPs structurally organize the ECM [237, 238] and regulate tumour cell proliferation through the regulation of angiogenic processes that are required for tumour development and cellular migratory processes that are also an intrinsic requirement for the establishment of tumour cell masses at remote sites. Lumican is associated with clinical outcome in cancer and appears tumour-specific [105]. Lumican specifically inactivates MMP-14, through which it suppresses ECM remodelling, angiogenesis and cellular migration which all contribute to an inhibition of tumourogenesis [90, 92, 93, 111, 122, 239–241].

As seen in Table 2.2, lumican is associated with a diverse range of cancer types and plays many functional roles in the affected tissues; however, the role of lumican in cancer varies with tumour type. Lumican is expressed and secreted by human melanoma cells but not by normal melanocytes [94]. Lumican binds to α2β1 integrin and inhibits melanoma cell adhesion [242]. Melanoma cell migration is also blocked by inhibiting MMP-14 [91]. Lumcorin, a peptide derived from lumican's ninth LRR repeat, is a potent MMP inhibitory peptide. Lumcorin inhibits tumour cell growth [95] and migration [93] through alterations in focal adhesion complexes [92]. Actin cytoskeletal organization has also been shown to be disrupted by lumican binding to α2β1 integrin in A375 melanoma tumour cells [241] and it also inhibits proliferation of B16F1 melanoma cells and lung metastasis [239].

Lumican also inhibits pancreatic tumour cell growth [97]. Lumican is expressed in alpha cells of pancreatic islets and pancreatic cancer cells [98]. Lumican stimulates growth but inhibits replication and invasion by human pancreatic cancer cells [99, 100] and in pancreatic ductal adenocarcinoma [101]. Lumican expression is also upregulated in lung adenocarcinoma and squamous cell carcinoma where it inhibits cell migration and cellular proliferation [114, 115] but is downregulated in giant cell bone tumours [50].

Overexpression of lumican upregulates gelsolin and filamentous actin reorganization [104] and is associated with a good outcome in Stage II, III colon carcinoma [105]. However, lumican expression in advanced colorectal cancer with nodal metastasis correlates with a poor prognosis [106, 107]. In osteosarcoma, lumican regulates tumour cell adhesion by modulating TGFβ2 activity [108] and is positively correlated with differentiation but negatively with the growth of human osteosarcoma cells [109]. In prostate cancer, an increase in lumican expression has been observed in the stromal tissue surrounding prostate primary tumours. In vitro experiments showed that lumican inhibited the migration and invasion of metastatic prostate cancer cells isolated from lymph node, bone and brain. A significant increase in prostate cancer cell invasion has been observed in the peritoneum of lumican knockout mice, demonstrating the inhibitory role lumican normally plays in the ECM preventing prostate cancer invasion [102].

Lumican significantly attenuates breast tumour cell functional properties, including proliferation, migration and invasion in vitro. Lumican also downregulates oestrogen receptor α/β expression in breast cancer cells suppressing the expression of major matrix effector molecules such as MMPs and EGFR which normally promote breast cancer progression [243]. Low lumican levels are associated with a poor prognosis in lymph node-negative invasive breast carcinomas [244].

Endometrial cancer is the most frequent type of malignant gynaecological tumour in the Western world with ~40,000 cases reported annually [119]. Lumican staining is more intense in

endometroid-type endometrial cancer than in endometrial intraepithelial neoplasia, although the functional roles of lumican in these tissues remain to be fully determined [120].

Lumican is a cytoplasmic and pericellular component of neuroendocrine tumours including carcinoid tumours and neuroendocrine cell carcinomas and their associated stromal tissues. Lumican is observed in the rough endoplasmic reticulum and neuroendocrine granules in neuroendocrine tumours as well as the interspaces between collagen fibres in stromal tissues and occurs in carcinoid tumours with a higher frequency than in neuroendocrine cell carcinomas [116]. High expression levels of lumican in these tissues are believed to explain the slow growth rates of such tumours. Schwannoma-like salivary pleomorphic adenomas are rare but are associated with chondroid tissue formation with the ectopic chondrogenesis driven by BMP-2. Pleomorphic adenomas are the most common form of salivary gland tumours. Lumican is predominantly found in the hyaline (100%) and fibrous regions (89.4%) and in chondroid masses in salivary pleomorphic adenomas [117].

Lumican is expressed in uterine cervical squamous cell carcinoma particularly at the periphery of cancer cell nests and by fibroblasts in proximity to these tumour cell masses but is not expressed by normal squamous or ductal cells close to these cancer cells [118]. The role of lumican in these tumours has not been determined; however, elevated lumican levels at the periphery of such cancer cell nests may regulate the growth or invasion of human cervical cancer cells [118].

2.7 Concluding Remarks

KS is an underappreciated GAG of considerable complexity. This chapter has attempted to outline the molecular recognition and information transfer properties that this biomolecule conveys to a diverse array of interactive KS-PGs and the multifunctional roles they have in cellular regulation. Not only is KS attached to an extensive array of PGs with diverse functional properties but it also decorates a number of mucin-like glycoproteins of importance in the tumour environment. The interactions that KS regulates are of importance in a diverse range of physiological processes in health and disease. A greater understanding of the KS glyco-code and how it is interpreted by different cell populations will undoubtedly pave the way to the elucidation of further complexities of this fascinating molecule and its participation in cellular regulation in health and disease and may be of application in repair biology.

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3 Hyaluronan in the Tumor Microenvironment

Fiorella Mercedes Spinelli, Daiana Lujan Vitale, Ina Sevic, and Laura Alaniz

Abstract

The extracellular matrix is part of the microenvironment and its functions are associated with the physical and chemical properties of the tissue. Among the extracellular components, the glycosaminoglycan hyaluronan is a key component, defining both the physical and biochemical characteristics of the healthy matrices. The hyaluronan metabolism is strictly regulated in physiological conditions, but in the tumoral tissues, its expression, size and binding proteins interaction are dysregulated. Hyaluronan from the tumor microenvironment promotes tumor cell proliferation, invasion, immune evasion, stemness alterations as well as drug resistance. This chapter describes data regarding novel concepts of hyaluronan functions in the tumor. Additionally, we discuss potential clinical applications of targeting HA metabolism in cancer therapy.

Keywords

Tumor microenvironment · Hyaluronan · Drug Resistance · Immune response · Stemness · Hyaluronan metabolism · Cancer Stem Cells · Cancer therapy · Hyaluronidases · Hyaluronan Synthases · Extracellular Matrix · Tumor-Associated Macrophages · CD44 · DNA damage · UGDH

3.1 Introduction

Hyaluronan (HA) is a member of the glycosaminoglycan family and is synthesized by all vertebrate organisms. HA is a key component of the extracellular matrix (ECM) of almost all mature tissues. It is present in the interstitial matrix, but is also detected at the intracellular level [47]. It is defined biochemically as an unsulfated linear molecule composed by repeating disaccharide units of D-glucuronic acid (GlcA) and D-N-acetylglucosamine (GlcNAc) joined by β(1–3) and β(1–4) linkages [47]. Despite its simple chemical structure, HA can interact with different cellular receptors (CD44, RHAMM, LYVE-1, TLR4), proteins (HA binding proteins: HABP), and proteoglycans (aggrecan, versican, neurocan, brevican). This allows HA to regulate a variety of biological processes such as cell growth, adhesion, migration, and differentiation. Even more, HA is known for having multiple and complex

F. M. Spinelli · D. L. Vitale · I. Sevic · L. Alaniz (✉)
Laboratorio de Microambiente Tumoral, Centro de Investigaciones Básicas y Aplicadas (CIBA), Universidad Nacional de la Pcia. de Bs. As. Centro de Investigaciones y Transferencia del Noroeste de la Pcia. de Bs. As. (CIT NOBA, UNNOBA-CONICET), Junín, Buenos Aires, Argentina
e-mail: isevic@comunidad.unnoba.edu.ar; ldalaniz@comunidad.unnoba.edu.ar

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physiological functions in wound healing, inflammation, and immune responses [121]. The HA metabolism is strictly regulated in physiological conditions and is a balance between the synthesis and degradation of this glycosaminoglycan. However, in the tumor ECM, expression and size of HA are dysregulated, conducting forward a microenvironment characterized by uncontrolled cell proliferation, invasion, immune evasion as well as drug resistance (Fig. 3.1).

In recent decades, the association between HA of the tumor microenvironment and cancer progression has been extensively studied. In the majority of malignancies, high levels of HA in stroma or within tumor mass itself are directly correlated with a poor prognosis of the disease, favoring the emergence of resistance to chemotherapy, and promoting the generation of aggressive tumor phenotypes [5, 59] as well as immunological response [122].

Therefore, the following sections highlight new advances and concepts of the importance of HA in tumor progression and its relationship with key mechanisms that define the tumor cell fitness, like drug resistance, immune response, DNA repair, and stemness control.

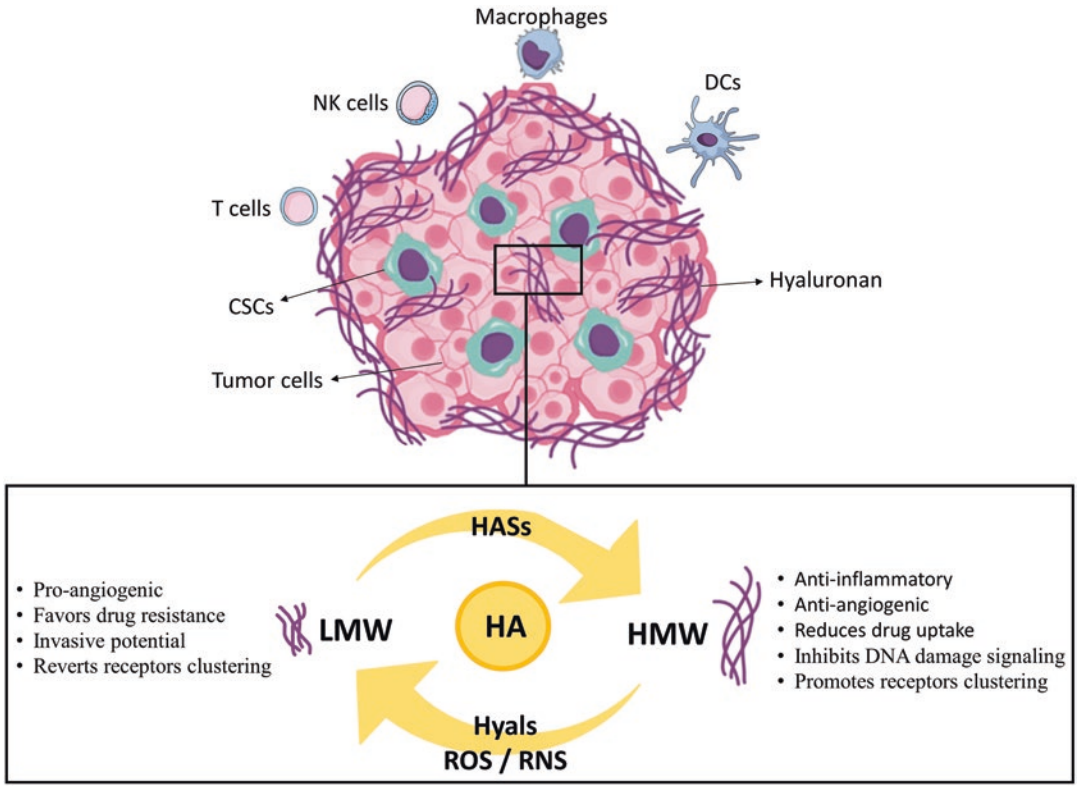

Fig. 3.1 HA metabolism and the tumor microenvironment. In tumor tissues, HA metabolism is strictly dysregulated as well as its interaction with binding proteins. HA biosynthesis is catalyzed by the action of different isozymes of HASs, localized at the internal surface of the plasma membrane. The amount and size of HA chains present in tumor context depends on the activity of Hyals, the enzymes responsible for its degradation. Also, HA can be fragmented into different molecular weight forms by ROS or RNS produced during cancer. This HA metabolism is crucial because HA biological functions depend on its molecular weight. Different-sized HA interacts and triggers different signals in other cells of the tumor microenvironment, like immune cells and CSCs. HA: hyaluronan; LMW: low molecular weight; HMW: high molecular weight; HASs: hyaluronan synthases; Hyals: hyaluronidases; CSCs: cancer stem cells

3.2 HA Metabolism in the Development of Drug Resistance

The activity of the enzymes that synthesize or degrade HA generates size-specific fragments with a different biological activity and with a specific role in tumor progression [146].

3.2.1 Altered HA Synthesis in the Tumor Microenvironment

HA biosynthesis is catalyzed by the action of different isozymes called hyaluronan synthases (HASs; HAS-1, HAS-2, and HAS-3), localized at the internal surface of the plasma membrane in eukaryotic cells. These isozymes require the cytosolic substrates UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc) to synthesize HA. The end of the growing chain displaces a UDP residue when the next sugar is added, while releasing the growing chain through the cell membrane to produce large HA polymers [141]. The study of the three HAS isoforms has discovered differences in the catalytic properties and the specific regulation for each isozyme, particularly in their ability to determine the HA size. HAS-1 and HAS-2 enzymes catalyze the synthesis of high molecular weight forms of HA (HMW HA), whereas HAS-3 drives the production of large amounts of low molecular weight chains (LMW HA). HAS-2 is the most commonly expressed isoform in normal conditions, whereas HAS-3 is a most active and predominant enzyme in pathological conditions, such as inflammation and cancer [55, 124].

Furthermore, the amount and size of HA chains present in tumor context depend on the activity of the enzymes responsible for the degradation of this glycosaminoglycan, called hyaluronidases (Hyal-1 to -4, PH-20/PSAM1, and the pseudogene HyalP1), being Hyal-1 and Hyal-2 the most characterized. In human tissues, Hyal-2 is mainly found at the cell surface, which, in combination with extracellular reactive oxygen/nitrogen species (ROS/RNS), cleaves HMW HA to produce an extracellular LMW HA of approximately 20 kDa [87]. On the other hand, Hyal-1 and Hyal-3 are present in the lysosomes together with glucosaminidases and glucuronidases, producing HA oligomers (tetrasaccharides) [27, 73]. Even more, it has been discovered that the transcript of Hyal-3 is widely expressed; however, it does not show a catalytic activity by itself and may have a role in the regulation of Hyal-1 expression [91].

HA products differentially affect several cancer hallmarks like proliferation, invasion, angiogenesis and drug resistance, and this is why HA metabolism is considered to be clinically relevant. Previous studies support the association between the regulation of HA synthesis and the expression of HAS enzymes with a poor prognosis in patients with cancer.

In ovarian cancer, it has been discovered that HAS-1 expression enhanced the presence of micro-vessels, and was negatively correlated with overall survival time. Similar results were observed in colon cancer, where high HAS-1 transcript levels were related to poor survival [148]. However, HAS-2 and HAS-3 expressions were unrelated to the survival time of the patients. Furthermore, the role of HAS enzymes was studied in different stages of the disease as well as previous and post-chemotherapy treatment. Yabushita et al. found that HAS-1 mRNA levels were higher in effusions compared to primary ovarian tumors and solid metastases; and even more, HAS-1 was overexpressed in pre-chemotherapy compared to post-chemotherapy effusions [147]. Besides, HAS-2 transcript was increased in solid metastases and primary carcinomas. Also, HAS-3 levels were overexpressed in primary ovarium carcinoma and effusions compared to solid metastases [142]. On the other hand, carboplatin treatment significantly increased the expression of HAS-2 and HAS-3 isoforms, and consequently, induced HA production in ovarian cancer tissues collected during chemotherapy and at recurrence [103]. HAS3 has also been described to play a crucial role in pancreatic cancer [71] altering mitotic spindle and epithelial organization [94, 105], promoting a malignant phenotype.

Another important aspect in the regulation of HA synthesis has been the use of 4-methylumbelliferone (4-Mu), a molecule capable of inhibiting the activity of HASs by depletion of cellular UDP–glucuronic acid and downregulation of HAS-2 and HAS-3 mRNA levels [70]. The role of 4-Mu as an anticancer agent was studied in a broad spectrum of tumors. In human pancreatic cancer cells, 4-Mu inhibited pericellular HA matrix formation and increased apoptosis [89]. On the other hand, it has been reported that the combination of different chemotherapy treatments with 4-Mu administration favors the response of chronic myeloid leukemia cells (K562). More specifically, 4-Mu treatment decreased cell proliferation and induced senescence in cells treated with vincristine [79]. In turn, the exposure of K562 cells to the combination of low doses of 4-Mu plus doxorubicin increased apoptosis and sensitivity to doxorubicin therapy [131]. Together, these results suggest that the treatment with 4-Mu favors the sensitivity of leukemic cells to chemotherapy by decreasing their HA-mediated resistance to apoptosis.

Experimental and clinical data similarly demonstrated that the amount of HA is higher in malignant epithelium and tumor stroma compared to its lower levels in healthy breast tissue [6]. Several experiments performed on different breast cancer cell lines revealed that HAS-2 mRNA is predominantly active in MDA-MB-231 and HS578T aggressive cell lines compared to the less aggressive MCF-7 [14, 48]. Additionally, Schwertfeger and collaborators found that the expression of HAS-2 was higher in bone metastases, indicating an important role of this isoform in cell invasion and breast cancer progression [113].

In mice and humans, HAS-2 expression is controlled through a post-transcriptional regulation by a long non-coding RNA (lncRNA) called HAS-2 antisense 1 (HAS2-AS1). It is a natural antisense transcript synthesized by the opposite DNA strand at the HAS-2 locus. It was described for the first time in osteosarcoma cells, where Chao et al. demonstrated that the overexpression of HAS2-AS1 suppresses HAS-2 expression by regulating mRNA levels [30]. Recently, different studies have investigated the role of HAS2-AS1 as a lncRNA with oncogenic functions in different types of tumors. In glioma cells, the knockdown of HAS2-AS1 inhibited cell viability, migration, and invasion through the PI3K/AKT signaling pathway [153]. Furthermore, high levels of the HAS2-AS1 were reported in oral squamous cancer cells under hypoxic conditions, which promote epithelial-to-mesenchymal transition (EMT), increasing their migratory and metastatic potential by stabilizing HAS-2 [154]. Similarly, it has been demonstrated that HAS-2 was overexpressed and mRNA levels were highly correlated to the expression levels of HAS2-AS1. Moreover, HAS-2 and HAS2-AS1 are tightly regulated during TGFβ-mediated EMT in breast cancer cells [65]. Taken together these results indicate that the lncRNA HAS2-AS1 regulates AH biosynthesis and might represent an indicator of an aggressive phenotype and prognosis factor.

A considerable amount of literature has emerged reporting the expression and the importance of hyaluronidase enzymes in tumor aggressiveness. In colorectal carcinoma, a decrease in Hyal-1 levels was found in serum samples from patients compared to healthy donors [64]. Other studies have shown an increased activity of multiple isoforms of Hyal, which differentially affected tumor cell growth and was mainly associated with aggressive stages of the disease [20, 56]. The overexpression of Hyal-1 also represents an accurate diagnostic marker for bladder and prostate cancer progression and recurrence [37, 69, 98]. Even more, it has demonstrated the importance of Hyal-1 in metastasis, where non-metastatic and low HA-expressing prostate tumor cells increased their metastatic potential when transfected with Hyal-1, allowing the dissemination to lymph nodes [68].

Furthermore, several reports emphasized the role of hyaluronidases in breast and ovarian cancer. Knockdown of Hyal-1 reduced tumorigenicity of MDA-MB-231 and MCF-7 human breast cancer cells, decreasing the invasion, proliferation, and xenograft tumorigenesis [116]. The upregulation of Hyal-1 expression seemed to promote cell growth and migration *in vitro* and *in vivo* [12, 125, 126]. Hyal-1 transcript levels and

its catalytic activity are increased in epithelial ovarian cancers associated with decreased expression of estrogen receptor ERα [152]. Another important aspect in the study of hyaluronidases in cancer is the ability of these enzymes to reverse multidrug resistance and alter cell-cycle kinetics in chemo-resistant carcinomas. For example, the addition of Hyal-1 inhibited glioblastoma stem cell gene expression and sensitized glioblastoma cells to temozolomide [46]. Other therapies using similar strategies are discussed in a separate section.

3.2.2 Size-Specific HA Fragments in Tumor Progression and Drug Resistance

HA biological functions depend on its molecular weight. HA fractions can be classified according to their molecular size into HA oligomers (oHA, <10 kDa), LMW HA (10–250 kDa), HMW HA, (>1000 kDa), and very high molecular weight HA (vHMW HA, >6000 kDa). vHMW HA is found only in a type of rodent, the naked mole rat [15, 130]. This type of rat has an increased longevity and null incidence of cancer development due to a higher concentration of HMW HA in its skin. Interestingly, these animals express a particular isoform of HAS-2 and have a reduction in the activity of hyaluronidases in their fibroblasts. This causes a massive expression of HMW HA that might lead to an extended cancer protection [15, 130].

Separately from the specific activity of Hyals, the "native" HMW HA can be fragmented into different molecular weight forms by ROS or RNS produced during tissue inflammation, injury, sepsis, or cancer. The role of LMW HA as a pro-angiogenic and tumorigenic mediator is well known, due to its ability to modify several signaling pathways [21, 150]. Whereas the endogenous HMW HA is known for its anti-inflammatory and anti-angiogenic properties [85]. The size of HA can differentially activate HA receptors, and consequently, trigger different signaling pathways. HMW HA forms have multiple binding sites on CD44 receptor and stimulate its clustering. However, it has been proposed that LMW HA and oHA may act as antagonists, altering the specific clustering activated by native HMW HA [144]. In this sense, numerous studies have highlighted the role of HA in several types of cancer. For example, the treatment of colon cancer cells with oHA reduced the expression and activity of cyclooxygenase-2 followed by a decrease in HA synthesis ([86]). In another study, it has been shown that LMW HA induced apoptosis of colorectal cancer cells, triggered activation of the immune system, and inhibited tumor growth [1]. In addition, it was proved that LMW HA concentrations are increased in interstitial tumor fluid of colorectal cancer patients. Also, this was associated with lymphatic vessel invasion by cancer cells and the formation of lymph node metastases [112]. Regarding breast cancer, it has been demonstrated that HA decasaccharides diminished the MDA-MB-231 cell growth, migration, invasion, and reduced HA expression by these cells [133]. These results were also confirmed in clinical studies where LMW HA expression was elevated in cancer cells with invasive potential [146]. Moreover, an excess in the stromal LMW HA has been shown to facilitate lymph node metastasis of melanoma cells [5, 36]. Furthermore, it has been proposed that oHA decreased proliferation, invasiveness, aggressiveness, and endogenous HA production in glioma [140], ovarian [119], and osteosarcoma cells [50].

Another important function of the different-sized HA on the tumor microenvironment is the ability to modulate the resistance to chemotherapy, one of the main causes of cancer recurrence. Several studies proposed that high deposition of HMW chains of HA in tumor stroma reduced the uptake of chemotherapeutic drugs, inhibited angiogenesis, and diminished the intracellular drug diffusion, favoring tumor growth [11, 99, 111]. In contrast, it has been demonstrated that oHA fragments sensitize tumor cells to chemotherapy in several tumor types, such as peripheral nerve sheath tumors [118], lymphoma [26], and chronic myeloid leukemia [78]. The proposed mechanisms include decrease in the activation of Akt/PI3K pathway, induction of the

internalization of CD44 and P-glycoprotein that finally leads to apoptosis. On the other hand, results from our group demonstrated that the co-treatment with LMW HA and doxorubicin favored the development of drug resistance in lymphoma T, osteosarcoma, and breast cancer cells, by activating survival pathways and promoting angiogenesis *in vitro* and *in vivo* [136].

3.2.3 HA Synthesis Is Implicated in Cell Metabolism: New Perspectives

The reorganization of the ECM that surrounds the tumor microenvironment plays a key role in cell-extrinsic metabolic regulation. Many of these processes are dependent on increased glycolytic metabolism, which can provide the energetic currency and biosynthetic substrates required for cell proliferation [33, 100]. Although ECM remodeling and elevated glycolysis are coincident in multiple biological contexts, the mechanistic links between these processes are not well established in cancer.

GAGs production, including HA, requires the cytosolic precursors UDP-GlcUA and UDP-GlcNAc, substrates of HAS enzymes as was described above. It has been discovered that these two UDP-sugars are implicated in the modulation of HA synthesis and aggressiveness in cancer cells ([92]). The UDP-GlcNAc is produced at the final step of the hexosamine biosynthetic pathway, and is necessary for post-translational modification of proteins by O-GlcNacylation [151]. This reaction affects cancer cells since it promotes key tumorigenic functions, such as regulation of cell proliferation signaling, resistance to apoptosis, and enhanced angiogenesis and invasiveness [45]. Moreover, O-GlcNacylation increases HAS-2 stability and regulates the localization and trafficking of HAS-3, which promotes HA expression and deposition at the cell membrane [34].

The other HAS substrate, UDP-GlcUA, is synthesized by the enzyme called UDP-glucose dehydrogenase (UGDH). This enzyme catalyzes the oxidation of UDP-glucose to form a molecule of UDP-GlcUA. The catalytic action of this enzyme generates two NADH molecules that can modify the NAD:NADH ratio [134, 135]. This, consequently, produces alteration in NAD levels and radically affects cell metabolism since NADH is crucial for mitochondrial activity [19]. UDP-GlcUA can act as a substrate of glucuronyltransferase (UGT) enzymes, responsible for several detoxification reactions [107]. Therefore, an increase in UDP-GlcUA amounts could lead to increase in HA production as well as favor an enhanced chemoresistance, typical of aggressive cancers.

In recent years, evidence has emerged regarding the implication of the UGDH enzyme in different types of cancer, and the association of its activity with tumor aggressiveness and progression. Hagiuda et al. studied the role of UGDH enzyme in lung adenocarcinoma, relating the expression and localization of this enzyme with the prognosis of the disease. The nuclear localization of UGDH was correlated with larger tumor size, lymphatic and vascular invasion, metastasis, as well as less survival compared to nuclear UGDH-negative lung adenocarcinoma patients [44]. Moreover, UGDH levels were increased in pancreatic acini samples from pancreas carcinoma patients [52]. Also, it was found that the inhibition of UGDH expression in HCT-8 colorectal carcinoma cells successfully decreased cell motility and cell aggregation, effects that were reverted by the addition of exogenous HA [139]. Preliminary results from our laboratory demonstrated that the silencing of UGDH enzyme with a specific siRNA increased intracellular epirubicin (EPI) availability in MDA-MB-231 breast cancer cells, without affecting their survival rate.

Tumorigenesis depends on the reprogramming of cellular metabolism. The first metabolic hallmark identified in tumor cells was an alteration in glucose cell metabolism, by a dysregulated balance from oxidative phosphorylation to aerobic glycolysis. This leads to higher glucose uptake and accumulation of lactate in tumor cells, even below normoxic environments, commonly known as the Warburg Effect [75]. Interestingly, in a recent study, it was confirmed that the

treatment of cells and xenografts with Hyal activated a steady increase in glycolysis pathways. In particular, the perturbation of HA from ECM induced the upregulation of the glucose transporter GLUT1 at the cell membrane, favoring glucose uptake in tumor cells and promoting cell migration. These results provided a long-established mechanistic link between HA and the metabolism of tumor cells [123].

3.3 HA Biology and the Tumor Immune Response

An important part of the tumor microenvironment is composed of immune cells. Usually, immune cells, in concert with malignant cells, play an important role in the modulation of the tumor microenvironment. Regarding HA, immune cells are able to bind HA and their response depends on several factors, such as the cell type, the molecular weight of HA, and the HABP present in the tumor milieu [121].

One of the most important immune cells of the tumor microenvironment is macrophages. Classically, it is well known that these cells can adopt different phenotypes: M1 (classically activated) and M2 (alternatively activated). M1 are referred to phenotypes driven by IFNγ, while "M1-like" include those that lead the antitumor responses. M2 are referred to macrophage phenotypes driven by IL-4 or IL-13, whereas "M2-like" include diverse phenotypes that promote tumor and evasion of effective immune response [109]. Macrophages can have different actions in cancer which depend mainly on the tissue and the stage of progression. Tumor-associated macrophages (TAMs) are M2-like macrophages play crucial roles in driving growth and progression [83]. In most tumor types, TAMs stimulate proliferation and migration of tumor cells, promote tumor angiogenesis, and remodel the microenvironment [29]. It was previously demonstrated that HMW HA induced an anti-inflammatory polarization, in comparison to LMW HA, which induced an inflammatory macrophage phenotype [108].

A histopathology study suggests that an increased number of macrophages correlated with HA accumulation in breast cancer tumors [129]. Also, it was previously shown that TAMs infiltrated into breast tumors in an HA-dependent manner and enhanced neovascularization and tumor growth [63]. We have demonstrated that the effect of HMW HA as an inductor of the angiogenic behavior of macrophages in breast tumor context is in part consequence of the presence of TNF-stimulated gene-6 (TSG-6) [122]. Taken together, these effects may be due to the action of HA and its biding molecules like TSG-6. TSG-6 allows HA to crosslink with other matrix components such as the serine protease inhibitor, inter-α-inhibitor (IαI) heavy chains [32]. This structure allows stabilization and structural integrity of the ECM, providing a scaffold for the mobilization of monocytes/macrophages [29]. Even more, this crosslink structure could activate monocytes/macrophages to release several growth factors and ECM remodeling components [29]. Therefore, the interaction of monocytes/macrophages with this altered ECM may induce protumoral changes in these cells.

Another type of cells of the tumor microenvironment, dendritic cells (DCs), are essential for inducing immunity against cancer. Immature DCs can present self-antigens to T cells, which leads to immune tolerance. These effects could be through T-cell deletion or the differentiation of T cells. Mature DCs can start the differentiation of T cells into effectors. DCs capture tumor antigens released from tumor cells and present them to T cells, contributing to tumor rejection [121].

It was previously demonstrated that HA fragments and oligosaccharides, but not HA HMW, induce maturation and activation of DCs *in vitro* and tumor context. For example, Rizzo et al. have demonstrated that HA as LMW improves human DCs maturation due to its ability to enhance their immunogenic properties as well as their migratory capacity toward lymph nodes [106]. Although DCs could respond to tumor antigens, the functions of these cells are altered by the tumor microenvironment [121]. Even though it is known that DCs express TSG-6 and IαI, it is not yet determined whether these cells can organize HA in cross-linked complexes [57, 80].

There are several subsets of T cells, like CD4+ (helper) and CD8+ (cytotoxic), that recognize antigens expressed in most tumor cells. Enhanced intratumor CD8+ T-cell infiltration has been described as a positive prognostic marker in melanoma, head and neck, breast, bladder, urothelial, ovarian, colorectal, prostatic, and lung cancers [38, 121]. Different populations of T cells can infiltrate tumor areas [8]. Among these, cytotoxic CD8+ memory T cells are usually capable of killing tumor cells and are associated with good prognosis [41]. These T cells are supported by CD4+ T helper 1 cells, by the production of several cytokines. Another type of CD4+ T cells are T regulatory cells (Tregs), which play an immunosuppressive role. These cells are often described as pro-tumoral [51] since they can inhibit the recognition and clearance of tumor cells by the immune system [23]. A tumor microenvironment with a high number of Tregs correlates with worse prognosis in many types of cancer [9, 28, 49]. *In vitro* studies have shown that activated T cells are induced to bind HA [35]. CD44–HA binding is believed to facilitate the rolling and extravasation of T cells to inflammatory sites [18]. Memory T cells differentiation into a Treg phenotype can be induced by HMW HA, but not low-MW HA [17]. Moreover, the addition of HA to Tregs enhanced Foxp3 expression and the production of IL-10 and IL-2 [16]. However, HA binding is also induced on a subset of nonregulatory CD4+ T cells, where its function has not been determined [39].

Natural killer (NK) cells are immune cells that show cytolytic activity against cells under stress such as tumor cells [93], and they are considered an excellent target to enhance during cancer immunotherapy. Physiologically, NK cells also secrete several cytokines after activation, like interferon-γ (IFN-γ), that can modulate the function of other innate and adaptive immune cells [93]. The antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism mediated by these cells and is an important strategy in antitumoral therapy. In this approach, the therapeutic antibody binds to the antigen expressed on the tumor cell surface, then the Fc region of the antibody is recognized by CD16 from NK cell inducing the tumor cell death. In tumor context, it has been observed that the high level of HA reduces NK cell accessibility and contributes to resistance to ADCC in tumor tissues in a xenograft model of ovarian cancer [117]. Moreover, CD44 is constitutively expressed by resting NK cells and stimulation of these cells leads to an upregulation and activation of CD44. It was previously demonstrated in NK cells that LMW HA plus IL-2, IL-12, or IL-18 could activate CD44 and promote IFN-γ synthesis [110].

3.4 HA Biology, DNA Damage, and Stemness Control

One of the cellular components of the tumor microenvironment (TME) is the cancer stem cells (CSCs), which play a very important role in tumor initiation, progression, and metastasis. CSCs have high plasticity and contribute to intratumoral heterogeneity, which is known to influence disease progression and therapeutic efficiency. They have the properties of self-renewal, cell death evasion, multidrug resistance, and metastasis, although they are known to be able to stay quiescent for extended periods of time. TME and CSCs have a complex cross-talk which adds to the plasticity of the tumor and also contributes to its heterogeneity [40, 97].

CSCs reside in specialized areas called niches. The stem cell niche is a specialized microenvironment consisting of cells that provide support and factors to maintain the stemness. These cells, through cell–cell contact, can anchor stem cells to the niche, which is crucial to maintain stem cells close to niche factors and also away from differentiation stimuli. On the other hand, CSCs can send signals to activate quiescent niche or to strengthen an already activated niche and by doing so to promote the expansion of the tumor [120, 138].

HA is an important part of tumor niches, which provides a favorable environment for tumor cells. It has been demonstrated that it can influence the behavior, differentiation, and self-renewal of stem cells in healthy tissue as well as CSCs. CSCs express a variety of cell surface

markers such as CD44, CD133, and CD24. CD44 is one of the HA receptors and is expressed by CSCs in a wide variety of cancers. In tumor tissue, and in combination with HA, it is strongly linked to generation, maintenance, and survival of CSCs; moreover, it plays an important role in maintaining the stemness [7, 138]. It has been shown in acute myelogenous leukemia (AML) that CD44 is the crucial regulator of leukemia stem cells (LSC) homing to the stem niches and maintenance of stemness. Also the molecular mechanisms that in normal cells maintain quiescence could facilitate LSC survival [66]. Growing evidence indicates that the interaction between HA and CD44 in the extracellular domain promotes multiple signaling pathways which play a critical role in tumor cell proliferation in a variety of solid tumors [127]. Numerous signaling molecules from Wnt, Hedgehog, and Notch pathways have been associated with niche control and are known to be stem cell activators and to accelerate tumorigenesis [120].

Epithelial-to-mesenchymal transition (EMT) can be reactivated in cancer. It promotes tumor progression by the functional loss of E-cadherin, which results in loss of cell polarity and consequently, loss of tissue organization. It provokes an increase in migration and invasion and inhibition of apoptosis and senescence, favoring stemness profile. Through the EMT transition, tumor cells tend to develop strong invasive and metastatic ability, which helps them to migrate to different sites using the circulatory system. The interplay between HA and CD44 in tumor tissue can result in the modification of ECM, which in turn can result in better support for tumor cell colonization. High HA expression is associated with the process of EMT, and CD44 has been described as a marker of EMT [7, 72, 149].

Mesenchymal stem cells (MSCs) can be integrated into the tumor microenvironment after recruitment and interact with ECM. Depending on the interaction between the MSCs and the tumor cells and the ECM, these cells can take a pro- or anti-tumor profile, which in turn leads to greater heterogeneity of the tumor tissue ([67, 84]). It has been shown that communication between cancer cells and MSCs can be accomplished through direct cell-to-cell interaction and paracrine interaction through signaling molecules by which cancer cells can recruit specific competent "naıve" MSCs from the adjacent tissue in the process of tumorigenesis and influence them to support tumor progression [13]. CD44, the main HA ligand, is also expressed as a cell surface receptor on MSCs. It has been shown that MSCs migration to the injured tissue depends on CD44 expression and its interaction with HA. It has been suggested that in Desmoid tumors, noncancerous growth within connective tissue, paracrine regulation of HA signaling may contribute to MSC recruitment and deregulated proliferation [22].

Tumorigenesis is usually associated with high levels of genetic instability. It has been suggested that most, if not all, tumors have some form of defect in DNA repair. Cellular DNA can be damaged by a variety of endogenous factors, like ROS, and exogenous factors, like UV light and ionizing radiation (IR). As a response to DNA damage, cells developed checkpoints which prevent advancement through the cell cycle and which can initiate DNA repair, activate transient cell cycle arrest or in the case of irreparable DNA damage, apoptosis. One of these pathways is DNA damage response (DDR), which continuously monitors DNA integrity [24, 81]. If the DNA repair mechanisms are damaged or dysfunctional, genomic instability arises. This event is marked as one of the hallmarks of cancer. Different cancers can have diverse etiologies and types of DNA damage, which leads to different DNA repair pathways being more active in those cancers [2]. Many cancers are resistant to radiotherapy in part because of the existence of quiescent CSCs; however, the fact that CSCs may exist within hypoxic niches that result in lower levels of ROS and higher free radical scavenging systems also increases the resistance. There are suggestions that, in glioblastomas, CSCs could be radioresistant in part due to upregulation of the DDR. Some tumors can display the overactivation of DDR kinases, which provides them with the ability to endure and survive DNA damage [3, 82].

When the DDR process occurs, one of the early events is the phosphorylation of the serine 139 of H2AX histone. This modification depends on the action of phosphatidylinositol 3-kinase (PI3K)-like family members. This family includes ataxia telangiectasia-mutated (ATM), AT-related (ATR), and DNA-dependent protein kinase (DNA-PK). H2AX also plays a central role in a hypoxic tumor microenvironment by promoting tumor phenotypes. It was shown that the regulation of HMW HA could attenuate ATM-H2AX pathway induced by exogenous oxidants [90, 145].

Hypoxia is a common event in the solid tumor microenvironment. Blood supply and oxygen levels are limited in proliferating cancer cells because of the lack of vasculature or the presence of anomalies in tumor vasculature. Normal hypoxic cells experience oxidative DNA damage and as a consequence, genetic aberration, which eventually leads to cell death. On the other hand, cancer cells evolve and adapt for hypoxic survival, and consequently acquire resistance to radiotherapy and invasive and metastatic ability [102]. HMW HA inhibits the H_2O_2-induced DNA damage signaling. There are also evidences that HA can reduce the formation of reactive oxygen species in MSCs [145]. Moreover, HA in a hypoxic niche, as well as in the stem cell niche, could offer the conditions to reduce oxidative DNA damage and sustain the quiescent state of these cells. In this manner, a dysregulation of HA expression might affect the niche, inducing stem cell alterations and stimulating tumor formation.

3.5 Potential Clinical Applications of Targeting HA Metabolism in Cancer Therapy

In the last years, several potential clinical applications of targeting HA family in cancer therapy have emerged. In this section, we will describe the most relevant.

3.5.1 Targeting HA Synthesis

As was mentioned before, several studies of different types of cancer indicate that there is an accumulation of HA in tumor tissues and that is why inhibiting HAS has been proposed as a way of diminishing HA levels. HAS1 expression was increased in the bladder, prostate, and renal cancers [31, 42, 77]. It was previously demonstrated that HAS1 knockdown in bladder cancer cells induced apoptosis, inhibiting both tumor growth and angiogenesis [43]. Also, HAS2 and HAS3 knockdown was found to inhibit tumor growth in breast and osteosarcoma [74, 77, 115, 132].

4-Mu is a well-known chemical inhibitor of HA synthesis [25, 60, 77, 88]. 4-Mu inhibits HA synthesis by competition, since it is glucuronidated by endogenous UGT [70]. Even more, it was found that 4-Mu diminished mRNA levels of HAS2 and HAS3 [70]. It was demonstrated, in several tumor types, that 4-Mu can inhibit tumor growth, proliferation, motility, invasion, and metastasis [4, 76, 77, 95]. In other words, 4-Mu is a non-toxic orally bioavailable dietary supplement that inhibits HA synthesis and has an antitumor effect. That is why this HA synthesis inhibitor has the potential for clinical use.

3.5.2 Targeting HA Degradation

Altering the degradation of HA has also been proposed as a way to target HA accumulation. As it was mentioned before, catabolism of HA is mediated by the HYALs. It is well known that the addition of HYALs to chemotherapeutics enhances the catabolism of HA, as well as significantly increases the efficacy of chemotherapeutics. This HYAL effectiveness of improving chemotherapies has been explored in several tumor types [10, 62, 96, 143]. *In vitro* and *in vivo*, the addition of HYALs decreased chemoresistance, increased drug penetration and cell death [143]. Recombinant HYAL is currently being investigated under different formulations for solid tumors. The PEGPH20, a pegylated formulation of a recombinant form of human HYAL

from Halozyme Therapeutics, Inc, is being applied in several clinical trials for different cancers in combination with other therapies, like biological, chemo- or radiotherapy (clinicaltrials.gov). The results of these trials indicate that intravenous administration induced the inhibition of tumor cell growth. Besides, the degradation of HA may result in the reduction of the interstitial fluid pressure, allowing better penetration of chemotherapeutic agents as well as immune cell into the tumor zone [128].

Moreover, several chemical compounds have been tested for targeting HA degradation. In this chapter, we will only discuss sulfated HA (sHA). sHA is a chemically modified HA that includes the presence of sulfate groups. It was demonstrated that sHA derivatives inhibit HYAL-1 [54]. sHA was found to induce apoptosis in the prostate cancer cell, inhibiting tumor growth [11]. Also, sHA exhibited antitumor activity in bladder cancer models by inhibiting proliferation, motility, and invasion [58]. Targeting HYALs could be an attractive strategy for inhibiting tumor growth in this tumor.

3.5.3 Targeting HA Receptors

CD44 is the most attractive HA receptor to target because it was found to be overexpressed in tumor cells and it is considered a stem cell marker. Targeting CD44 in cancer therapies has been tried by different strategies: (i) DNA vaccines, (ii) anti-CD44 monoclonal antibody, and (iii) nanoparticle delivery of CD44siRNA [77, 101, 114, 137, 155]. However, a phase I analysis trial with CD44v6 targeting in head and neck squamous cell carcinoma showed severe adverse reactions, skin toxicity with a fatal outcome which led to the termination of the development program [104]. Therefore, the approach of targeting CD44 will need to be carefully evaluated before being using it in cancer therapy.

3.5.4 HA as a Drug Delivery Vehicle

It is well known that HA has excellent biocompatibility, biodegradability, and non-immunogenicity [53]. Furthermore, HA has the ability to bind to cell surface receptors, like CD44, that is overexpressed in tumor cells. It can simultaneously bind to multiple copies of CD44, leading to an enhanced activity. As it was mentioned before, CD44 is considered as a marker for CSCs. Because of all these reasons, HA is considered as an ideal drug delivery vehicle, particularly for cancer drugs [53]. HA can be directly conjugated to antitumor drugs or can be used in several types of nanomaterials like micelles and hydrogels [61]. HA nanomaterials are attractive systems for the effective delivery of chemotherapy drugs.

In this context and taking into account the studies described in this chapter, HA arises as a modifiable therapeutic target, which in combination with different antitumoral treatments could be a new and useful strategy to improve cancer therapies.

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Fibronectin in the Tumor Microenvironment

4

Peter Qiao and Zheng-Rong Lu

Abstract

Fibronectin (FN) is a large glycoprotein that plays a diverse set of biological roles. This chapter discusses the structural biology, the normal biological functions, and the molecular role of FN and its splice variants in cancer cell proliferation, metastasis, and chemoresistance. The potential role of FN in cancer imaging is discussed in detail. The chapter also discusses the future directions of basic and translational research of fibronectin in the context of the tumor microenvironment and its role in tumor biology.

Keywords

Fibronectin · Extradomain B fibronectin · Extradomain A fibronectin · Cancer · Extracellular matrix · Epithelial-to-mesenchymal transition · Fibroblasts · Metastasis · Prognosis · Imaging · Integrin · Tumor microenvironment · Wound healing · Splice variants

P. Qiao · Z.-R. Lu (✉)
Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA
e-mail: zxl125@case.edu

4.1 Introduction

Fibronectin (FN) was first described in chicken embryo fibroblasts transformed with oncogenic viruses in 1975. It was subsequently termed the large external transformation-sensitive protein due to the reduction of expression observed in cells infected with virus [1–3]. The importance of this new molecule was unclear, but its discovery spurred a revolution in how the components of the extracellular matrix (ECM) were thought to interact with cellular processes.

FN is a large glycoprotein with size ranging from 230 to 270 kDa due to alternative splicing of the FN1 gene that codes for the FN monomer. The FN monomer consists of three structural motifs: type I, type II, and type III repeats [4, 5]. These motifs enable the wide array of biological functions attributed to FN [5]. Type I and type II repeats are stabilized by intra-repeat disulfide bonds, whereas the type III repeat adopts a β-barrel configuration [4, 6, 7].

FN contains several protein domains, each responsible for a specific function (Fig. 4.1). The first five type I repeats near the N-terminus of the FN molecule are critical to the *in vivo* formation of FN dimers, as well as binding to other macromolecules such as fibrin, heparin, and tumor necrosis factor alpha [8–10]. This polyvalent binding domain is adjacent to the collagen binding domain composed of both type I and type II domains which enables the attachment of FN to

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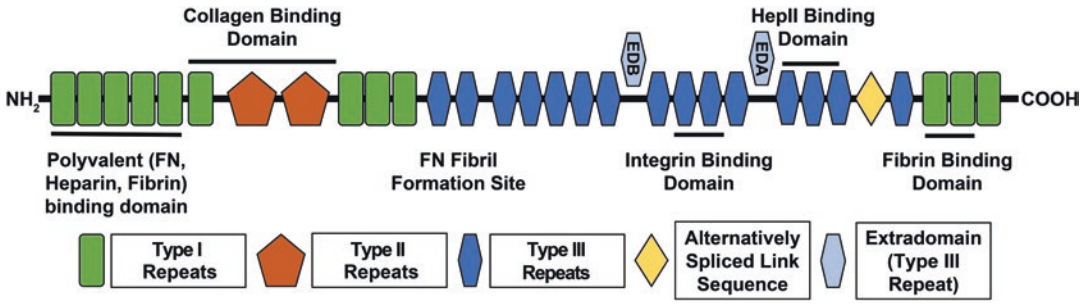

Fig. 4.1 Structure of fibronectin (FN) monomers. Key domains governing intermolecular interactions of FN with other ECM components and cellular components are highlighted. Alternatively spliced extradomains found in oncofetal FNs are included in their spliced locations. In the ECM, the monomeric FN molecule interacts with nearby FN molecules and other ECM proteins to form large, polymeric structures that support cell growth and wound healing. Malignant cells can exploit and alter the normal functions of FN to support tumor growth and disease progression

collagen I fibrils [11]. The first two type III domains are critical for the formation and stabilization of FN fibrils [5]. Crucially, FN contains domains that allow FN to interact with cell surface receptors. The integrin binding domain is composed of a variable number of alternatively spliced type III domains that contain the key Arg-Gly-Asp (RGD) motif well established as the binding site for a variety of integrin cell surface receptors [12].The integrin binding domain is stabilized by the PHSRN sequence found elsewhere in FN [13]. A binding site for the syndecan family of cell surface receptors is found within the HepII domain near the C-terminus. At the C-terminus itself, cysteine residues form interchain disulfide bonds that stabilize the FN dimer [14]. Several sites are alternatively spliced within the FN molecule. Two type III repeats can be alternatively spliced, between the 7th and 8th type III repeats for the EDB extradomain, or between the 11th and 12th type III repeats for the EDA extradomain. A linking sequence between the 14th and 15th type III repeats can also be variably spliced, with several subsequent splice variants generated [5]. The wide variety of functional domains and alternative splicing enable the FN protein to interact with a wide variety of other ECM components and cell surface receptors that enable the diverse biological functions of this key ECM component.

4.2 Role of Fibronectin in Normal Microenvironment

Fibronectin is found in both the ECM and blood plasma. Plasma FN is a soluble protein produced by hepatocytes and found in the bloodstream, where it plays a role in the formation and stabilization of fibrin clots and early tissue repair [15, 16]. Circulating plasma fibronectin stabilizes blood clots by crosslinking to fibrin clots via coagulation factor XIIIa in damaged tissues [3]. Cellular fibronectin is found in tissue ECM and is synthesized by a wide variety of cell types, including fibroblasts and endothelial cells [17]. The roles that cellular FN plays in the normal tissue microenvironment are diverse. Cellular FN is deposited by migrating fibroblasts and endothelial cells during repair of damaged tissue. Cellular FN scaffolds allow for the migration of cells via integrin cell surface receptors, stimulation of collagen deposition, and binding of wound healing-promoting factors heparin sulfate and hyaluronic acid [18–20]. Cellular FN is also an important cue for differentiation of stem cells within both damaged and healthy tissues, where it can trigger developmental programs through binding and stimulation of integrin cell surface receptors [21]. Cellular FN functions as a mechanotransducer, enabling the ECM to respond to external forces by stimulating or inhibiting ECM remodeling

factors such as fibroblasts, metalloproteinases, and transglutaminases [22, 23].

4.3 Role in Tumor Microenvironment

4.3.1 Role in Tumor Proliferation

Fibronectin supports tumor growth primarily through the activation of cell surface integrin receptors. Early attempts to study FN in the context of malignancy correlated expression with angiogenesis [24] and proliferation [25] through immunohistological analysis. These studies found FN was expressed in the walls of tumor-associated vasculature and in regions of high cell proliferation of rat livers exposed to carcinogenic compounds. However, the biochemical role of FN was still opaque until the identification of cell receptor binding motifs within the FN molecule. This breakthrough led to the discovery of FN-mediated activation of the integrin family of cell surface receptors. Integrin cell surface receptors enable cells to respond to changes in the extracellular environment [26]. Identified by the combination of α and β subunits from which they are assembled, integrins have been frequently implicated in supporting the proliferation of tumor cells through activation of a variety of anti-apoptotic and proliferative pathways commonly activated in cancer, including the Akt and MAPK pathways [26, 27]. The integrin binding amino acid sequence RGD found within the integrin binding domain of FN enables interaction with a wide variety of integrin subunits. FN has been shown to bind to the pro-proliferation integrin αvβ1 and subsequently activate the Akt and Ras pathways [28]. The Akt pathway appears to be a major effector of FN-associated proliferation through induction of mTOR and subsequent activation and proliferation of genes and inhibition of tumor suppressor proteins through stimulation of integrin α5β1 [29, 30] (Fig. 4.2). Ultimately, the stimulation of the Akt pathway by FN is capable of affecting the function of dozens of signaling molecules downstream of the Akt protein, resulting in changes in cellular metabolism, chromatin remodeling, and cell cycle regulation that support an increased rate of tumor growth [31]. Increasing expression of FN predicts larger human primary tumors, presence of lymph node metastasis, higher grade, and poorer disease-free and overall survival [32].

4.3.2 Role in Cancer Invasion and Metastasis

FN plays a complex and important role in cancer invasion and metastasis through the induction of the epithelial-to-mesenchymal transition (EMT). FN has been shown to activate EMT in cancer cells, which has been implicated in the development of a more invasive and metastatic phenotype in a variety of cancers [2].The shift of cells to a mesenchymal phenotype is considered an important step in invasion of tumor cells into nearby tissue and metastasis to distant organs. Through adoption of a mesenchymal phenotype, it is thought that cancer cells become better suited to survive detachment from the ECM and invasion into the systemic circulation [33]. FN activates a variety of cell signaling pathways, such as focal adhesion kinase (FAK) [34], SNAIL-related zinc-finger transcription factor (SLUG) [35], signal transducer and activator of transcription 3 (STAT3) [36], and ERK/MAP [37], to trigger EMT and subsequently enhance the metastatic potential of the cancer cells in a variety of tumors. Cells grown in FN-enriched media exhibit upregulation of EMT markers N-cadherin and vimentin and morphological changes consistent with EMT [34]. Activation of FAK by FN is mediated by the integrin family of cell surface receptors, with which FAK is closely associated. Phosphorylated FAK is then cleaved by the calpain-2 protease, leading to activation of EMT [34]. Similarly, activation of SLUG, STAT3, and ERK/MAP pathways is also mediated by integrin family proteins (Fig. 4.3).

FN expression may act to induce EMT at the mRNA level as well. A recent study has shown that FN mRNA may act as "competitive endogenous RNA" [39]. Bioinformatic analysis of the FN mRNA revealed a binding site for the

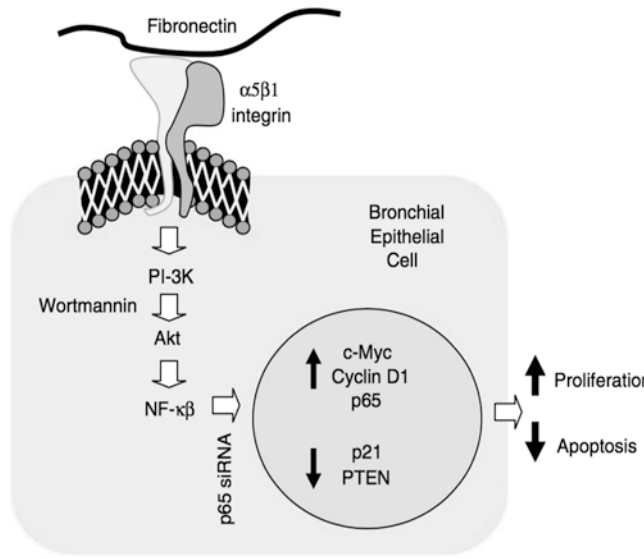

Fig. 4.2 Fibronectin and integrin mediate signal transduction and key intracellular signaling pathways. Activation of the α5β1 integrin by FN triggers PI-3K phosphorylation, triggering a signaling cascade that increases the proliferation of bronchial epithelial cells and reducing the expression of pro-apoptotic genes. Adapted from Han & Roman [30]

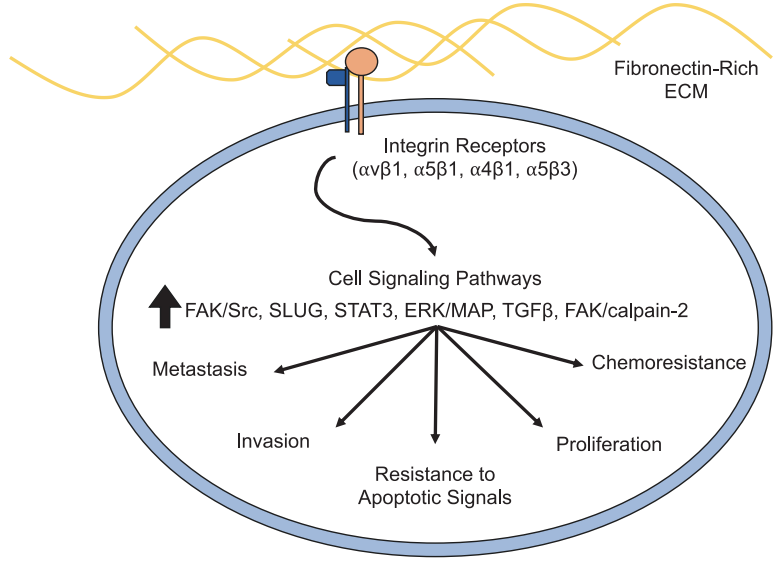

Fig. 4.3 Downstream effectors of FN activation of integrin cell surface receptors. FN activation of integrin transmembrane proteins is capable of activating several canonical and non-canonical cell signaling pathways that contribute to the development of a highly aggressive cancer cell phenotype. Adapted from Wang and Hielscher [38]

regulatory microRNA miR200c in the 3′ untranslated region. In the absence of FN mRNA overexpression, miR200c inhibits the expression of several regulatory proteins responsible for initiating EMT. FN mRNA overexpression in malignant cells results in the binding and sequestration of miR200c, reducing the availability of miR200c to inhibit EMT. Knockdown of FN mRNA through siRNA treatment resulted in a marked decrease in EMT markers and reversal of EMT *in vitro*.

FN can also influence the metastatic potential of cells without induction of EMT. A recent study of pancreatic cancer metastasis demonstrated that exosome-mediated upregulation of FN in liver Kupffer cells induced formation of a pre-metastatic niche and enhanced metastasis of cancer cells [40]. Overexpression of FN was induced by circulating tumor exosomes prior to the metastasis of cancer cells, suggesting FN does not merely interact with the primary tumor to enable metastasis but also primes distant

tissue to become a receptive metastatic niche for the growth of new tumors. Tumor cells can also induce FN overexpression in other cell types found in and around the tumor to produce a microenvironment primed for metastasis and invasion [41]. In a model of ovarian cancer, tumor-associated mesothelial cells were found to overexpress FN that enabled metastasis of cancer cells through tumor cell-mediated stimulation with transforming growth factor beta (TGF-β) and subsequent activation of the downstream signaling molecules RAC-1 and SMAD2/3 [42].

FN promotes invasion and metastasis through EMT-dependent and EMT-independent pathways. FN is a strong activator of the EMT program that enables tumor cells to achieve the metastatic phenotype and also primes both local tumor and distant pre-metastatic sites to better support the migration, invasion, and metastasis of a wide variety of cancers.

4.3.3 Role in Tumor Chemoresistance

Chemoresistance is a well-documented phenomenon whereby tumors treated with cytotoxic agents gain resistance to the therapeutic agent ultimately leading to treatment failure. Exposure of cells to an FN-rich microenvironment causes activation of several pathways that enable tumor cells to survive exposure to chemotherapeutic agents. *In vitro* study of breast and ovarian cell lines demonstrated that adhesion to FN resulted in increased Akt2 phosphorylation and subsequent increase in resistance to docetaxel treatment through inhibition of pro-apoptotic factors and upregulation of the pro-survival gene survivin [43]. FN can activate integrins α5β1 and α4β1 to increase the chemoresistance of myeloma, myeloid leukemia, and B-cell malignancies by increasing the ability of cancer cells to transport doxorubicin out of the cytoplasm via the drug transporter ABCC1, allowing the cells to grow despite the presence of large doses of chemotherapeutic drugs [44]. Lung cancer cells also gain a chemoresistant phenotype when cultured with FN, demonstrating an increase in activity in the Erk and Rho pathways and gaining resistance to etoposide, cisplatin, and doxorubicin [45, 46]. FN stimulation of the β1 integrin subunits resulted in activation of protein tyrosine kinase (PTK) and inhibited the activation of caspase-3, a critical mediator of apoptosis in all human cells [45]. Exposure to FN-rich microenvironments also decreases the expression of cell cycle inhibitor p21 and the stimulation of cell cycle promoter cyclin D1, enabling cancer cells to ignore apoptotic signals and continue division in the presence of chemotherapeutic agents [46]. In pancreatic cancer cell lines, increased expression of FN in tumor-associated pancreatic stellate cells induced chemoresistance to gemcitabine in pancreatic cancer cells through activation of the ERK1/2 pathway, protecting cells from the pro-apoptotic effects of gemcitabine [47]. Overall, FN expression stimulates a diverse set of signaling molecules to increase the resistance of tumor cells to pro-apoptotic signals while simultaneously increasing expression of genes driving cell division and drug efflux. Ultimately, these effects diminish the efficacy of systemic chemotherapy in malignancy.

4.3.4 Role of Fibronectin Splice Variants

The posttranscriptional diversity of FN mRNA further expands the relationship of FN and malignancy. FN is expressed as a family of several splice variants, both in the developing embryo and the adult. Early reports suggested that FN splice variants that contain the extradomain B (EDB-FN) or extradomain A (EDA-FN) type III domains, termed oncofetal fibronectins due to their expression during fetal development, are re-expressed aberrantly in malignancy [48, 49]. Expression of oncofetal FN in malignant tissue was first reported in 1990 in human breast tumors [49]. Expression of EDB-FN cor-

related with intermediate- and high-grade lesions, while it remained undetectable in normal and benign tumors [49]. Soon after this discovery, additional studies detected EDB-FN in multiple cancer types, including lung and intestinal tumors [48]. More recent studies corroborate these initial findings, demonstrating strong expression of EDB-FN and EDA-FN in the tumor microenvironment, originating from malignant cells, as well as tumor-associated endothelial cells [50]. Inclusion of the EDB domain in FN produces extensive changes in the tertiary structure of the FN monomer, as well as the formation of novel head-to-tail FN dimers not observed in other biochemical studies of the FN molecule [51]. These observations suggest that oncofetal FNs may serve functions divergent from those of normal FNs. The role of oncofetal splice variants in cancer is currently an active area of investigation. Definitive roles of EDB-FN and EDA-FN in the context of cancer biology are needed to be elucidated. However, substantial data suggest that EDB-FN and EDA-FN do play a relevant role in tumor malignancy and tumor microenvironment.

Single cell gene expression profiling of ex vivo glioblastoma cells revealed that EDB-FN expression is elevated in endothelial and vascular smooth muscle cells, supporting the hypothesis that EDB-FN may play a role in the formation of tumor vasculature. The study also discovered that EDB-FN acts as a modulator of transforming growth factor beta (TGF-β) signaling in glioblastoma-associated vasculature, encouraging vessel remodeling and upregulation of FN expression, providing a molecular basis for the role of EDB-FN in cancer [52]. EDB-FN is upregulated in both malignant and tissue stem cells after induction of EMT, establishing EDB-FN as a potential biomarker for this important pro-metastatic process [53]. EDA-FN interacts with integrin α5β1 to increase bone marrow production of myeloid-derived suppressor cells (MDSCs). The increased production of MDSCs was shown to increase tumor growth, and EDA-FN-exposed MDSCs decreased apoptosis and expression of anti-inflammatory and anti-cancer molecule arginase-1 [54].

4.3.5 Clinical Relationship of Fibronectin to Diagnosis and Prognosis

Tumor-associated FN overexpression has demonstrated the promise in diagnosis and prognosis of a variety of tumors. Analysis of FN expression in human esophageal squamous cell carcinoma samples revealed a strong correlation of increased stromal FN expression to lymphatic metastasis, poorer overall survival, and shorter progression-free survival [55]. Immunohistochemical study of 110 breast cancer samples from primary tumors demonstrated that FN expression could be correlated to tumor size, grade, and the development of distant metastasis [56]. Analysis of tissues from pancreatic ductal adenocarcinoma demonstrated that high FN expression correlated with larger, more advanced tumors [57]. Some evidence suggests that elevated levels of FN in body fluids may predict prognosis in acute lymphoblastic leukemia, although the general picture of FN in risk stratification is less clear in non-solid tumors overall [58].

The overexpression of FN in malignancy has been leveraged as a tool for medical imaging and cancer diagnosis. Antibodies and engineered antibody fragments capable of recognizing the expression of EDB-FN in the tumor were reported as early as 1997, with the use of *in vivo* models of mouse teratoma [59], as well as in positron emission tomography (PET) studies of astrocytoma and melanoma [60]. Antibodies capable of targeting EDA-FN *in vivo* were also reported [61]. In these early studies, targeting of oncofetal FN demonstrated favorable targeting properties of both antibodies and engineered antibody fragments identified through phage display. Later studies of EDB-FN binding antibody fragments in murine teratomas corroborated these early studies using a variety of antibody fragments and radionuclides for PET imaging [62, 63].

Targeted contrast agents have also been developed for magnetic resonance imaging (MRI) of FN- and EDB-FN-rich tumors. MRI with the targeted contrast agents provides high-resolution images of the expression of the biomarkers in solid tumors. Initial efforts focused on the

possibility of targeting fibrin-fibronectin complexes commonly formed in the angiogenic tumors using oligopeptide-targeted gadolinium-based MRI contrast agents [64]. Targeted MRI contrast agents specific to EDB-FN were developed using a small peptide ZD2 for more specific tumor imaging with MRI. Small peptides as targeting agents are advantageous over bulky antibodies and their derivatives because of the rapid clearance of the peptides from the circulation and low background noise for biomedical imaging. In addition, the small size of the peptide-targeted gadolinium-based contrast agents allows rapid and complete excretion from the body after the diagnostic imaging, which is an essential requirement for clinical translation. MR molecular imaging (MRMI) with the ZD2-targeted contrast agents has demonstrated the ability to detect aggressive tumors, e.g., triple-negative breast cancer, and distinguish between high- and low-risk prostate cancer [65–67] (Fig. 4.4a, b). ZD2-targeted contrast agents generated high contrast enhancement in tumors generated from aggressive cell lines, whereas tumors generated from less aggressive cell lines did not. The ZD2 peptide has also been used in designing PET probes for sensitive cancer imaging. The use of EDB-FN targeting of a ^{64}Cu PET probe demonstrated high specificity in a model of prostate cancer (Fig. 4.4c), showing strong accumulation of the radiotracer construct within prostate cancers and enabling the identification of slow-growing low-risk tumors from those likely to be highly aggressive [68].

4.4 Future Trends and Directions

4.4.1 Interactions Between Fibronectin and Tumor-Associated Stromal Cells

Current studies of FN and its role in the tumor microenvironment have focused on the interaction of FN with cell surface integrin receptors. While the research remains active and valuable, the field has begun to investigate the effect of FN overexpression in cancer through other mechanisms. As our understanding of the tumor microenvironment has improved, the interactions of FN with non-malignant cells found in the microenvironment, termed tumor-associated stromal cells (TASCs), have been more closely scrutinized. Cancer-associated fibroblasts (CAFs) are one such example of TASCs that interact with FN. Although CAFs do not share the hallmarks of malignant cells, a significant proportion of the cells are found within a growing tumor [69]. CAFs alter the tumor microenvironment through secretion of ECM components, ECM remodeling, and production of soluble factors that alter the biology of tumor cells [69, 70]. It is now becoming clear that CAFs are at least in part responsible for the abundance of FN found in the tumor ECM. CAFs form parallel "tracks" of FN that exhibit a high degree of directionality at sites of local invasion, organized through contractile forces generated by myosin II fibers within the cell transduced to FN fibers through the α5β1 integrin receptor [71]. Subsequently, cancer cells can invade along these directional FN fibril tracts through αv integrin subunit binding [71]. FN expressed by cancer cells can also transform non-malignant cells to adopt an anchorage-independent and pro-tumorigenic phenotype. Crosslinked FN molecules can be horizontally transferred between cancer cells in microvesicles and have been shown to activate the Akt and Erk pathways to drive anchorage-independent growth and survival in normal epithelial and fibroblasts [72]. Oncofetal splice variants also appear to play a role in the regulation of CAFs. Expression of oncofetal fibronectin splice variant EDA-FN can induce the differentiation of mouse embryonic fibroblasts into activated CAFs and subsequently increase the migration of breast cancer cells [73].

FN can also influence the behavior of pericytes, TASCs associated with angiogenesis and cancer progression [74]. While the role of FN is less defined in this cell population, there is nevertheless evidence suggesting pericytes may support tumor angiogenesis and metastasis. Analysis of tumor tissues taken from orthotopic models of brain cancer and melanoma identified a nestin and neuron-glial 2 chondroitin sulfate proteoglycan (NG2) double-positive pericyte

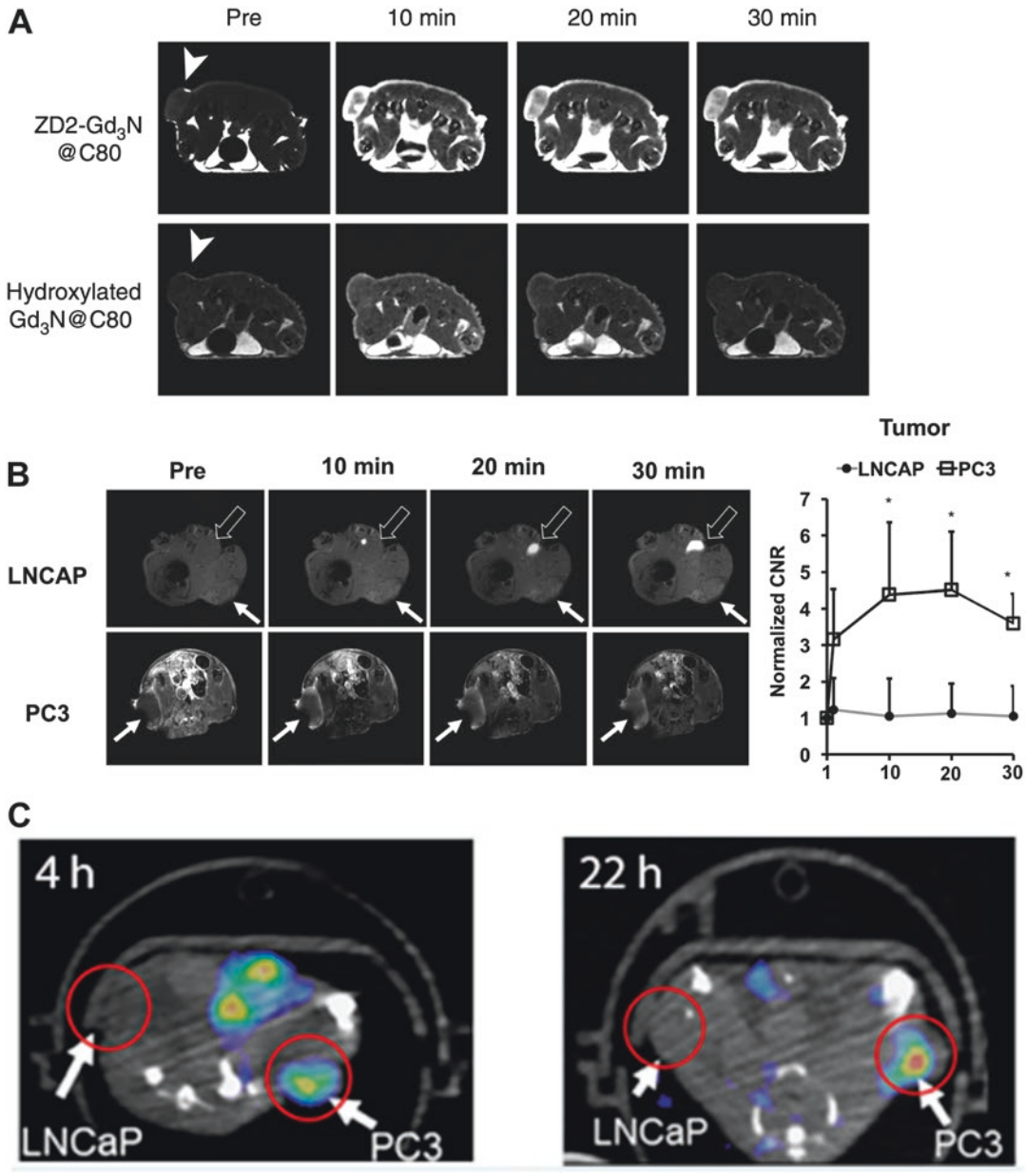

Fig. 4.4 (**a**) MRMI images of mice bearing MDA-MB-231 breast cancer tumors (arrowheads) after injection with ZD2-targeted hydroxylated Gd_3N@C80 specific to EDB-FN (top row) and non-targeted hydroxylated Gd_3N@C80 (bottom row) [67]. (**b**) MRMI images of mice bearing low-risk (LNCAP) and high-risk (PC3) prostate cancer tumors with ZD2-N_3-Gd(HP-DO3A) (white arrows). Increased contrast-to noise ratio is observed in the high-risk PC3 model [66]. Accumulation of MRMI agent is also observed in the urine (black arrows). (**c**) PET-CT images of mice bearing prostate cancer tumors (arrows and circles) after administration of a ZD2-targeted PET probe. Differentiation between high-risk (PC3) and low-risk (LNCaP) tumors is evident 4–22 h after probe administration [68]

subpopulation. The nestin$^+$/NG2$^+$ population was shown to be recruited to form neovasculature, with the intratumoral pericyte population consisting nearly exclusively of nestin$^+$/NG2$^+$ cells [75]. Another study demonstrated that pericytes can revert to a more pluripotent state through increased expression of Klf4, leading to formation of FN-enriched metastatic niches in the lung. The fate of pericyte cells was traced using a fluorescent reporter gene system, leading to the observation of pericyte activation in lung tissue of mice bearing tumors. Subsequently, the activation of pericytes was linked to the expression of FN and *in vitro* adhesion of cancer cells [41].

4.4.2 Fibronectin as a Therapeutic Target

FN is also becoming a popular target for therapeutic intervention. Due to the restricted expression of EDB-FN and EDA-FN in normal adult tissues, attempts have been made to use them as the basis for cancer vaccines. The EDB and EDA fragments have been conjugated to immunogenic proteins and injected into mice, with the expectation that such fusion proteins may overcome self-tolerance to these oncofetal FNs. In mouse models, this strategy has generated certain therapeutic response with EDB vaccination diminishing the size of fibrosarcoma tumors and EDA-FN vaccination reducing the growth of both primary and metastatic tumors in a model of metastatic breast cancer [76, 77]. FN targeting or mimetic strategies have also been used to improve drug delivery to tumors. Liposomes decorated with fibronectin-mimetic peptides bind to colon carcinoma cells by targeting the α5β1 integrin receptor on cancer cells [78]. Other approaches utilize the EDB-FN binding aptides to home liposomes to deliver a combination of siRNA and doxorubicin to reduce tumor growth [79]. The liposomes demonstrated enhanced accumulation and drug delivery to tumor sites, as well as significant antitumor effect [79]. Similar strategies have been used to target a liposome-encapsulated doxorubicin construct with high specificity to tumors generated from glioma cells via binding of engineered aptides to EDB-FN-overexpressing tumors [80]. Antibody and antibody fragments specific to EDB-FN have been used to deliver radioisotopes for cancer radioimmunotherapy of solid tumors. Conjugates of iodine-125 and indium-111 to the L19 anti-EDB-FN antibody and its antibody fragment resulted in prolonged animal survival and reduced tumor growth after a single injection in a teratocarcinoma model [81].

4.5 Summary

FN is a large and functionally diverse component of the ECM that plays important roles in tumor cell proliferation, migration, invasion, drug resistance, and metastasis. Overexpression of FN within the tumor microenvironment originates from tumor cells and tumor-associated stromal cells as a result of complex interactions with and between these two cell populations. FN acts through a wide diversity of integrin receptors as well as at the RNA level to drive proliferation, metastasis, and resistance to antineoplastic therapies. FN overexpression in the tumor microenvironment can be leveraged as a target for drug delivery as well as a biomarker in cancer detection and imaging through a variety of peptide- and antibody-based approaches. Future trends may lead to the investigation of FN's role in non-integrin cell signaling and in mRNA biology and the applications of FN in drug delivery and imaging.

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Matrix Metalloproteinases' Role in Tumor Microenvironment

5

Georgina Gonzalez-Avila, Bettina Sommer, A. Armando García-Hernández, and Carlos Ramos

Abstract

Cancer cells evolve in the tumor microenvironment (TME) by the acquisition of characteristics that allow them to initiate their passage through a series of events that constitute the metastatic cascade. For this purpose, tumor cells maintain a crosstalk with TME non-neoplastic cells transforming them into their allies. "Corrupted" cells such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs) as well as neoplastic cells express and secrete matrix metalloproteinases (MMPs). Moreover, TME metabolic conditions such as hypoxia and acidification induce MMPs' synthesis in both cancer and stromal cells. MMPs' participation in TME consists in promoting events, for example, epithelial-mesenchymal transition (EMT), apoptosis resistance, angiogenesis, and lymphangiogenesis. MMPs also facilitate tumor cell migration through the basement membrane (BM) and extracellular matrix (ECM). The aim of the present chapter is to discuss MMPs' contribution to the evolution of cancer cells, their cellular origin, and their influence in the main processes that take place in the TME.

Keywords

Acidosis · Adipocyte · Angiogenesis · CAFs · EMT · Hypoxia · Lymphangiogenesis · Mast cells · Metastasis · MMPs · TAMs · TANs · TME · TIMPs · "Warburg effect"

Abbreviations

5-FU	5-Fluorouracil
ADAMS	A disintegrin and metalloproteinases
ADAMTSs	ADAMs with thrombospondin motifs
ADFs	Adipocyte-derived fibroblasts
AM	Adrenomedullin
AP-1	Activator protein-1
AR	Androgen receptor
ASCs	Adipocyte/stromal stem cells

G. Gonzalez-Avila (✉) · A. A. García-Hernández
Laboratorio de Oncología Biomédica, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

B. Sommer
Departamento de Investigación en Hiperreactividad Bronquial, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

C. Ramos
Laboratorio de Biología Celular, Departamento de Fibrosis Pulmonar, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico

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BASCs	Bronchio-alveolar stem cells
bFGF2	Basic fibroblast growth factor-2
BM	Basement membrane
BMDMCs	Bone marrow-derived mesenchymal stem cells
bmMSCs	Bone marrow mesenchymal stem cells
CAAs	Cancer-associated adipocytes
CAFs	Cancer-associated fibroblasts
CCL2	Chemokine C-C motif ligand 2
CM	Conditioned medium
COX2	Cyclooxygenase 2
CSCs	Cancer stem cells
CXCL12	C-X-C motif chemokine 12
CXCR4	Chemokine receptor-4
DFSP	Dermatofibrosarcoma protuberans
ECM	Extracellular matrix
ECs	Endothelial cells
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
endMT	Endothelial-mesenchymal transition
ENO-1	Enolase-1
Epo	Erythropoietin
ER	Estrogen receptor
EREG	Pan-HER ligand epiregulin
ERK	Extracellular signal-regulated kinase
Et-1	Endothelin-1
ETP	Endotrophin
ETS	E-twenty-six-1
EVs	Extracellular vesicles
FASLG	FAS ligand
FIH-1	Factor-inhibiting HIF-1
FOXC2	Forkhead box protein C2
FoxP3+	Recruit forkhead box P3
Fru-2,6-P2	Fructose 2-6 biphosphate
FSP-1	Fibroblast-secreted protein-1
FZD	Frizzled
GPI	Glycosylphosphatidylinositol
GSCs	Glioma stem cells
HA	Hyaluronic acid
HGF	Hepatic growth factor
HIFs	Hypoxia-inducible factors
HSCs	Hematopoietic stem cells
Hsp-90	Heat shock protein-90
HuVECs	Human umbilical vein endothelial cells
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IL-2Rα	Interleukin-2 receptor α
JNK	c-Jun N-terminal kinase
KDR	Kinase insert domain receptor
KIF1B	Kinesin-like protein 1B
KitL	Kit ligand
KLF8	Kruppel-like factor 8
LECs	Lymphatic endothelial cells
LLC	Lewis lung carcinoma
LN	Lymph node
LOX1	Lectin-type oxidase LDL receptor 1
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein (LDL) receptor-related protein 1
LVs	Lymph vessels
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
MAPK	Mitogen-activating protein kinase
MaSCs	Mammary stem cells
MCP-1	Monocyte chemotactic protein-1
MCs	Mast cells
M-CSF	Monocyte colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MICA	MHC class I-related chain molecules A
MM	Multiple melanoma
MMPs	Matrix metalloproteinases
MMRN2	Multimerin-2
MMT	Mesenchymal-mesenchymal transition
MSLN F	Mesothelin
MT-MMPs	Membrane-type MMPs
NE	Neutrophil elastase
NF-κB	Nuclear factor κB

NGAL	Neutrophil gelatinase-associated lipocalin
NGF	Nerve growth factor
NHE1	Na+/H+ exchanger 1
NK	Natural killer
NKG2D	Natural killer group 2D
NRP-2	Neuropilin-2
NSCLC	Non-small cell lung cancer
OPN-MZF1	Osteospondin-myeloid zinc finger 1
PAI	Plasminogen activator inhibitor
PAR-1	Protease-activated receptor-1
PDA	Pancreatic ductal adenocarcinoma
PDGF-BB	Platelet-derived growth factor-BB
PDGFRβ	Platelet-derived growth factor receptor β
PEA-3	Polyoma enhancer activator protein-3
PECAM-1	Platelet endothelial cell adhesion molecule-1
PF4	Platelet factor-4
PFK-1	Phosphofructokinase-1
PFK-2/FBPase-2	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PGK1	Phosphoglycerate kinase-1
PI3K	Phosphoinositide 3-kinase
PPARγ	Peroxisome proliferator-activated receptor-γ
Rac	Rho-related C3 botulinum toxin substrate
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL5)
ROCK	rhoA-dependent kinase
ROS	Reactive oxygen species
SCCs	Squamous carcinoma cells
SCF	Stem cell factor
SCs	Stem cells
SDF-1	Stromal cell-derived factor 1
sE-Cad	Soluble E-cadherin
SERPINE1	Serine protease inhibitor E1
sKitL	Soluble kit ligand
Sp-1	Specific protein-1
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TCF	T-cell factor
TECs	Tumor endothelial cells
TFPI-2	Tissue factor pathway inhibitor-2
TGFβ	Transforming growth factor-β
TIGAR	TP53-induced glycolysis and apoptosis regulator
TIMPs	Tissue inhibitors of metalloproteinases
TME	Tumor microenvironment
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11 b
TNFα	Tumor necrosis factor-α
TXF/LEF1	T-cell factor/lymphoid enhancer factor 1
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
VSMCs	Vascular smooth muscle cells
ZEB1	Zinc finger E-box-binding homeobox 1
α-SMA	α-Smooth muscle actin

5.1 Introduction

Neoplastic cells from solid tumors are the consequence of a dynamic evolutionary process in which cells acquire characteristics that allow them to survive in a stressful microenvironment until they detach from the primary tumor and disseminate to create a metastatic colony [1]. Moreover, tumor cells at the metastatic site face a new tissue microenvironment in which they must develop strategies to subsist. Thus, to ensure the evolution of cancer cells, a complex interplay among neoplastic cells and the surrounding microenvironment is established, transforming this niche into their own tumor microenvironment (TME) [2]. Molecules, such as growth factors, chemokines, cytokines, structural and non-structural extracellular matrix (ECM) proteins, and basement membrane (BM) components, as well as different types of cells are part of

the TME [3]. Likewise, changes in TME metabolic circumstances such as nutrient decrease and hypoxic and acidic conditions drive tumor cells' development [1]. Moreover, epithelial-mesenchymal transition (EMT), anoikis (apoptosis resistance), angiogenesis, and lymphangiogenesis are processes that take place in the TME during cancer progression. Furthermore, the TME from the primary tumor participates in the preparation of the pre-metastatic niche in a distant tissue [4].

The matrix metalloproteinases (MMPs) are TME proteins with an important participation in cancer evolution. These enzymes can modify several BM and ECM elements; they also release and activate growth factors, cytoskeletal proteins, chemokines, adhesion molecules, and cytokines [5]. Furthermore, MMPs are not only released to the extracellular medium but are also located in different cell organelles such as mitochondria, nucleus, cell membrane, cytoplasmic vesicles, or specific granules [6]. Due to the functions and ubiquity of MMPs, they participate in all cancer progression steps, and therefore, they are considered as prognostic markers and therapeutic targets.

Because of the relevant role played by MMPs in cancer, the aim of this chapter is to review how MMPs contribute to tumor cells' preparation in the TME to continue with the next steps of the metastatic process. Particularly, this chapter points out the interaction between TME biochemical conditions and MMPs' expression, the processes that take place in the TME in which MMPs are involved, and the association between different TME non-neoplastic cells and MMPs' functions.

5.2 The Matrix Metalloproteinases (MMPs)

5.2.1 MMPs' Basic Molecular Structure

MMPs are endopeptidases that depend on zinc and calcium ions for their enzymatic activity. So far, 28 MMPs have been characterized in vertebrates, of which 24 are present in humans. According to their structural organization and substrate specificity, MMPs are grouped into collagenases, gelatinases, matrilysins, stromelysins, glycosylphosphatidylinositol-anchored MMPs, transmembrane type I and II, and other MMPs (Fig. 5.1) [7].

Most MMPs share a basic protein structure that consists of an amino-terminal signal peptide, a pro-peptide that contains the PRCGXPD sequence that includes the cysteine-SH group linked to a zinc ion at the catalytic site, a catalytic cleft, a proline-rich linker region, and a carboxy-terminal hemopexin-like motif that participates in enzymatic regulation and substrate specificity (Fig. 5.2). Besides this basic structure, other domains are present in MMPs; for example, membrane-type MMPs (MT-MMPs) sometimes have a furin recognition site between the pro-peptide and the catalytic center that participates in the intracellular activation of MMP zymogen forms [6]. Likewise, fibronectin and vitronectin motifs are part of the gelatinases (MMP-2 and MMP-9) and MMP-21 catalytic domains, respectively [6]. Similarly, MMP-9 contains a type V collagen-like region.

MMPs are synthesized as zymogens with the exception of MMP-23 that has no pro-peptide sequence. The pro-peptide "cysteine switch" with the zinc ion at the catalytic center forms a sphere-like structure that prevents substrate binding [8]. Conformational changes in the pro-domain modify the cysteine switch causing a partial activation of the enzyme. This first step of the pro-enzyme activation may be provoked by a proteolytic cleavage of the pro-peptide by trypsin, other MMPs, or plasmin, by the substrate binding to MMP exosites (sites outside the catalytic domain) that induces an allosteric activation, and by the chemical modifications that directly affect the thiol-zinc link produced for example by reactive oxygen species (ROS). Then the MMP removes the pro-peptide by an autocatalytic process to obtain full enzymatic activity [5]. Pro-MMPs can be also activated through their furin site by furin-like convertases and by forming activation complexes (see below).

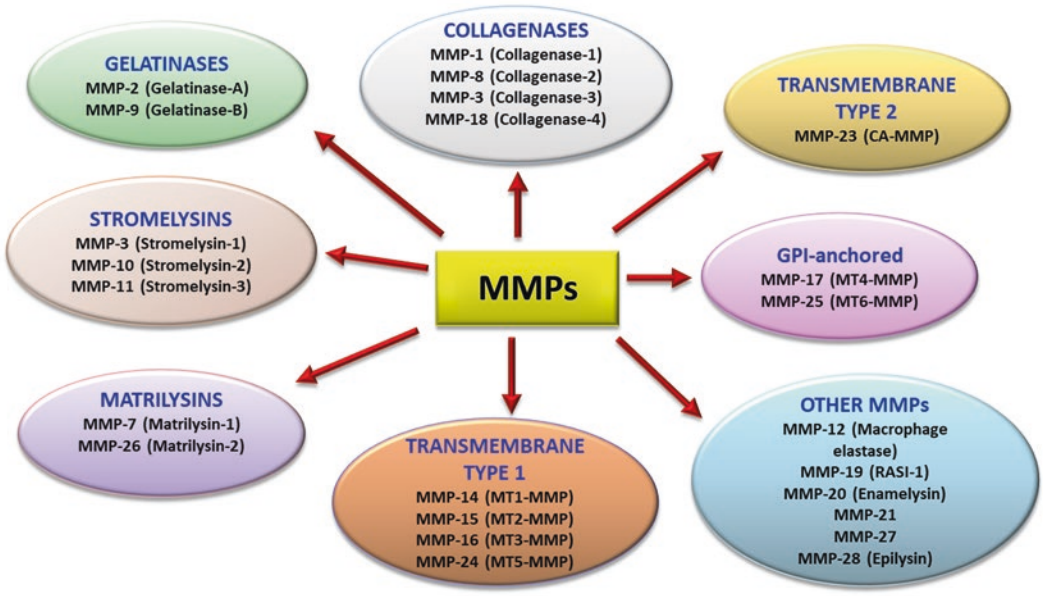

Fig. 5.1 MMPs' classification. Human MMPs have been categorized according to their molecular structure and substrate specificity into different groups: collagenases, gelatinases, stromelysins, matrilysins, transmembrane type I, transmembrane type II, GPI-anchored, and others. GPI glycosylphosphatidylinositol, MMPs matrix metalloproteinases

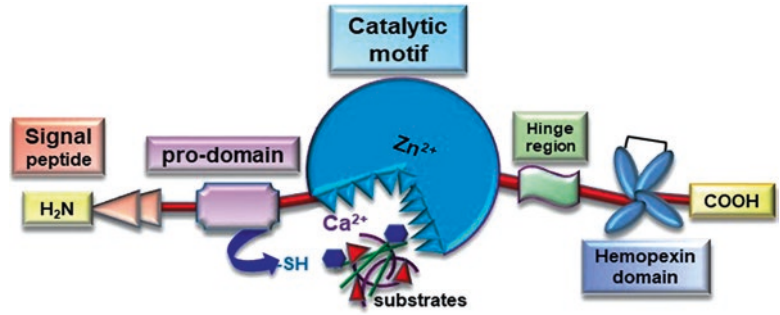

Fig. 5.2 Basic molecular structure of active MMPs. Domain structure is organized in most MMPs in a signal peptide, a pro-peptide that contains a cysteine that binds to a zinc ion in the catalytic motif that maintains the enzyme in an inactive form, a catalytic region that requires zinc and calcium ions for its activity, a hinge motif rich in proline, and an hemopexin domain

5.2.2 MMPs' Enzymatic Activity Regulation

MMPs are involved in many physiological events such as inflammation, embryogenesis, wound healing, neurite growth, and immunity, but a dysregulation in their enzymatic activity may cause tissue damage as seen in many non-cancerous diseases such as fibrosis, rheumatoid arthritis, epidermolysis bullosa, and aortic aneurysm [9]. Therefore, the enzymatic activity of MMPs must be strictly controlled by the tissue inhibitors of metalloproteinases (TIMPs). The inhibitory capacity of TIMPs is located at the N-terminal region that chelates the zinc ion from the MMP catalytic cleft forming a 1:1 stoichiometric

complex [10, 11]. The C-terminal domain is involved in the formation of MMP activation complexes. Besides MMPs' inhibition, TIMPs also block the enzymatic activity of a disintegrin and metalloproteinases (ADAMs) and ADAMs with thrombospondin motifs (ADAMTSs). Four different TIMPs have been identified: (1) TIMP-1, a 28-kDa glycosylated protein that can inhibit ADAM-10 and most MMPs except some MT-MMPs; (2) TIMP-2, a 21-kDa non-glycosylated protein that blocks MMPs and ADAM-12 enzymatic activity; (3) TIMP-3, a glycosylated molecule with a molecular weight of 24/27 kDa, capable of inhibiting the activity of MMPs, ADAMs, and ADAMTs; and (4) TIMP-4, a 22-kDa non-glycosylated protein that interferes with the activity of MMPs and ADAM-17, ADAM-18, and ADAM-33 (Table 5.1). Interestingly, TIMPs have other functions besides inhibiting the enzymatic activity of MMPs. For example, TIMP-2 participates in the activation of pro-MMP-2 through the formation of the pro-MMP-2/TIMP-2/MMP-14 complex. Briefly, two MMP-14 molecules dimerize in the cell surface and then the N-terminal region of TIMP-2 binds to the catalytic center of one MMP-14 followed by the binding of the hemopexin pro-MMP-2 region with the C-terminal domain of TIMP-2. The free MMP-14 cleaves the pro-peptide and the active MMP-2 is released [12]. This pro-MMP-2 activation mechanism is carried out in the neoplastic cell invadopodia during the invasion process. Likewise, TIMP-1 also forms the pro-MMP-9/TIMP-1/MMP-3 activation complex in which a large MMP-3 stoichiometric amount is necessary to saturate TIMP-1 and activate pro-MMP-9 [13]. The ability of TIMPs to bind to other ligands enables them to participate in processes such as apoptosis, cell proliferation, and angiogenesis, besides ECM turnover [11, 14].

5.2.3 Control of MMPs' Expression

MMPs are regulated at the genetic level by growth factors, glucocorticoids, cytokines, retinoic acid, and interleukins [15]. MMPs have three different types of promoter sites: (1) promoters with an activator protein-1 (AP-1) (~70 bp) and a TATA box (~30 bp) with the presence of a polyoma enhancer activator protein-3 (PEA-3) binding site located upstream, (2) promoters with a TATA box but no AP-1 site, and (3) promoters with no TATA box but with multiple GC boxes that are attaching zones for specific protein-1 (Sp1) and Sp3. For more details, see Yan C and Boyd DD [15].

Epigenetic processes, such as histones H3 and H4 acetylations that stimulate the synthesis of MMPs and promoter cytosine methylation in CpG that blocks MMPs' expression, are other ways to control the production of MMPs [15]. Likewise, regulation of MMPs' synthesis is mediated by the stabilization and destabilization of the transcripts with the participation of trans-acting RNA-binding proteins as well as several microRNAs at the post-transcriptional level [6].

5.3 TME Metabolic Conditions

TME is constituted by all the elements that surround the neoplastic cells and includes different types of cells, ECM components, secretory vesicles, exosomes, and signal molecules, all of which are immersed in particular metabolic conditions that drive cancer progression.

Table 5.1 TIMPs' molecular characteristics

Feature	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MW (kDa)	28	21	24/27	22
N-glycosylation site	2	0	1	0
Localization	Soluble/cell surface	Soluble/cell surface	ECM/cell surface	Soluble/cell surface
Pro-MMP interaction	Pro-MMP-9	Pro-MMP-2	Pro-MMP−2/−9	Pro-MMP-2

MW molecular weight, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of metalloproteinase

5.3.1 Extracellular Microenvironment Acidification and MMPs

Genetic mutations involved in tumorigenesis may provoke changes in the glucose metabolism of cancer cells. Warburg's first studies demonstrated that neoplastic cells increase their glucose uptake changing from an aerobic to an anaerobic glycolysis even though O_2 concentrations suffice for cell requirements [16]. This is the so-called Warburg effect. The molecular mechanisms involved in this tumor metabolic change are not well known, but it has been pointed out that mutations in the phosphoinositide 3-kinase (PI3K)/Akt pathway are implicated in glucose transporter Glut1 recycling, internalization, and activity augmenting its presence at the cell membrane [17–19]. The increase in glucose intake, together with a rise of hexokinase-2, phosphofructokinase-1 (PFK-1), and PFK-2 activities caused also by mutated PIK3, favors the anaerobic glycolysis with the subsequent lactate acid production and acidification of the TME [19]. Likewise, loss of p53 functions favors the Warburg effect since this molecule stimulates the expression of *T*P53-induced glycolysis and apoptosis regulator (TIGAR) that degrades fructose 2-6 biphosphate (Fru-2,6-P_2) because of its similarity with the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) phosphatase domain [20]. Fru-2,6-P_2 low levels provoke a decrease in the PFK-1 activity and an increase in the FBPase-1 action causing the inhibition of the glycolysis pathway. This change in glucose metabolism protects DNA from damage due to ROS produced during aerobic glycolysis [20].

The excessive production of lactic acid decreases the TME pH to around 6.4~7.0 [21]. TME acidification provokes an increase in cell membrane protrusions such as filopodia and invadopodia as well as lysosomes and exosomes trafficking to these structures allowing the location of MMPs and cathepsin B on the cell membrane. Moreover, pro-MMP-2 activation is carried out by cathepsin B instead of the classical mechanism in which the zymogen requires the pro-MMP-2/TIMP-2/MMP-14 complex for activation [22]. Further, it has been confirmed that this cysteine protease is able to activate pro-MMP-2 in HT29 colon cancer cell invadopodia in acidic conditions. Therefore, an increase of TME acidification due to lactic acid secretion or by the Na^+/H^+ exchanger 1 (NHE1) increases MMP-2 activity through cathepsin B action [21, 22]. Likewise, an increase of active MMP-2, MMP-9, and cathepsin B secretion in invadopodia in MDA-MB-231 metastatic breast cancer cells under acidic conditions has been observed [23]. Moreover, the NHE1 located in the invadopodia creates an acidic extracellular microenvironment suggesting a role of NHE1 in pro-MMP-2 and pro-MMP-9 activation [23]. Similarly, other membrane structures are affected by acidic conditions. Such is the case for caveolae that are dynamic invaginations of the cell membrane involved in signal transduction, endocytosis, and mechanical stress protection [21]. Caveolae have cathepsin B binding protein S100A10, plasminogen activator receptors, and plasminogen receptors [21]. Moreover, one of the caveolae plasminogen receptors is enolase-1 (ENO-1) that contributes to the Warburg effect through the downregulation of oxidative phosphorylation [24]. Interestingly, caveolae contain NHE1 and the voltage-gated sodium channel (Na_V) 1.5 that, besides controlling H^+ efflux, enhances NHE1 function increasing acidic conditions in the pericellular environment and cathepsin B activation [25]. In addition to cathepsin B's participation in pro-MMPs' activation, the presence of the urokinase plasminogen activator receptor (uPAR) in caveolae favors the activation of plasminogen to plasmin and in turn the activation of pro-MMPs [26]. Interestingly, ENO-1 indirectly participates in pro-MMP-2 and pro-MMP-9 activation since this enzyme interacts with plasminogen, uPA, and uPAR in neoplastic cells' surface favoring cell invasion [27].

5.3.2 Hypoxia and MMPs' Expression in the TME

The excessive proliferation of cancer cells together with a deficient blood supply produces regions with low pO_2 in solid tumors [28]. Levels below 10 mmHg of O_2 favor tumor cells' anaerobic glycolysis contributing to the TME acidification. In response to these metabolic conditions, a change in the gene expression pattern of neoplastic cells is produced [29]. Such is the case of the overexpression of the hypoxia-inducible factors (HIFs) [28]. HIFs are transcription factors composed of an HIF-1α or HIF-2α subunit and a constitutive HIF-1β chain [30]. HIF metabolism is regulated by O_2 concentrations. When O_2 levels are normal, Pro-402 and Pro-564 from the HIF-α subunits are hydroxylated and the subunits bind to the von Hippel-Lindau (VHL) E_3 ubiquitin ligase complex for their degradation by the ubiquitin proteasome system. In contrast, when O_2 concentrations are low, HIF-α subunits translocate to the nucleus where they dimerize with HIF-1β chain and form a complex with the coactivator CBP/P300. This complex is responsible of the expression of many proteins from the glycolysis pathway and glucose transporters as well as proteins synthesized during angiogenesis and the metastatic processes [30, 31]. HIF transcription activity can be blocked by its binding to the factor-inhibiting HIF-1 (FIH-1) that interferes with the interaction among the coactivator and HIF-1. Additionally, there is another α-subunit called HIF-3α [32]. This α-chain has different isoforms with different functions according to their structure and tissue location. For example, some full-length HIF-3α act as transcription activators in hypoxic conditions while others compete with HIF-1α and HIF-2α for binding to HIF-1β when HIF-1β is not enough. Likewise, some truncated HIF-3α isoforms behave as negative regulators of HIF-1α and HIF-2α, and other truncated variants have constitutive transcription functions [32].

On the other hand, neoplastic cell invasion increases during hypoxic conditions and therefore the possibility that HIFs are involved in the regulation of MMPs' expression. For instance, HIF-1α controls MMP-9 expression in MDA-MB-231 breast cancer cells and regulates MMP-15 transcription in pancreatic cancer cells, non-small lung cancer cells, and cervical cancer cells [33, 34]. Likewise, experiments done with HIF-1α siRNA demonstrated a downregulation of MMP-2 and MMP-9 expression together with a decrease in glioma cell migration capacity under hypoxic conditions [35]. Moreover, MMP-1 and MMP-3 synthesis was controlled by HIF-1α in bone marrow mesenchymal stem cells (bmMSCs) [36]. Furthermore, MMP-2 upregulation and E-cadherin downregulation was observed in hypoxic conditions induced by the use of cobalt chloride in esophageal cancer cells in which HIF-1α control was also involved [37]. Additionally, HIF-1α can upregulate MMP-13 expression in cells and in exosomes from nasopharyngeal carcinoma cells [38]. Likewise, an increase of MMP-7, MMP-14, and E-twenty-six-1 (ETS-1) synthesis but not of MMP-2 was detected in HepG2 and Hep3B hepatoma cell lines in hypoxic conditions [39]. However, this increase was independent of HIF-1α regulation since their expression was neither affected by the HIF-1α inhibitor TX-402 nor by the use of the HIF-1α-dominant negative vector (pHIF1αDN) suggesting an independent HIF-1α pathway for the transcription regulation of these genes. It is interesting to note that ETS-1 is a transcription factor that controls the synthesis of different MMP genes even though the increase observed in EST-1 expression in this study was not accompanied by the rise of other MMPs, for example, MMP-1, MMP-9, and MMP-13, that are augmented in ovarian and nasopharyngeal cancer cell lines [40, 41].

Likewise, while hypoxia determines MMP-14 expression in hepatoma cells, low O_2 concentrations have no effect on this MMP's synthesis in breast cancer cells. Moreover, hypoxia induces MMP-14 translocation to invadopodia due to its effects on the small GTPase rhoA in these cells [42]. Additionally, MMPs' expression could be regulated indirectly by HIF-1α. Such is the case of MMP-1 synthesis controlled by the chemokine receptor-4 (CXCR4) and MMP-17 whose transcription is induced by Slug (also known as

Snail2); the expression of both molecules is regulated by HIF-1α [43, 44].

5.4 MMPs' Induction of Epithelial-Mesenchymal Transition

Under TME pressure, neoplastic cells develop migration capacities through their transformation from well-differentiated cells into mesenchymal-like cells [45]. This process is known as epithelial-mesenchymal transition (EMT). During EMT, epithelial cells lose their intercellular interactions such as the adherent junctions in which the main protein is E-cadherin [46]. E-cadherin extracellular domain forms dimers that bind to E-cadherin dimers from other cells while the C-terminal intracellular region links to actin filaments through catenins such as β-catenin [46]. The expression of E-cadherin is directly or indirectly downregulated by the transcription factors zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, forkhead box protein C2 (FOXC2), Kruppel-like factor 8 (KLF8), E47, Snail (also called as Snail-1), Slug, and Twist involved in EMT [47]. In this context, the expression of ZEB1, ZEB2, Twist, Snail, and Slug genes is upregulated by MMP-14 [48]. Therefore, when MMP-14 is increased, mesenchymal markers such as N-cadherin, vimentin, and fibronectin are augmented. MMP-9 has also been implicated in EMT induction. Inhibition of MMP-9 expression using siRNA or the MMPs' inhibitor GM6001 decreases vimentin and fibronectin expression as well as the migration capacity of highly invasive A433 cervical carcinoma cells [49]. Moreover, MMP-9 knockdown also reduced Snail expression, whereas downregulation of Snail diminished MMP-9 expression together with a diminution of the mesenchymal marker synthesis and of the invasion ability. These results point out the existence of a regulatory loop between MMP-9 and Snail expression. Furthermore, loss of E-cadherin expression in A549 non-small cell lung cancer (NSCLC) cells increases epidermal growth factor receptor-(EGFR)-mitogen activating protein kinase/extracellular signal-regulated kinase (MAPK/ERK, also known as MEK/ERK) activity with an increase of ZEB1 and MMP-2 expression [50]. Interestingly, downregulation of ZEB1 provokes a decrease in MMP-2 synthesis and in cell invasion abilities [50]. Likewise, Snail expression can be regulated by ROS. In this context, SCp2 mouse mammary epithelial cells cultured with exogenous MMP-3 express Rho-related C3 botulinum toxin substrate (Rac)-1b, an isoform from Rac-1. Rac-1b in turn releases ROS to the cytosol. ROS induce the expression of Snail and therefore the downregulation of E-cadherin and the upregulation of EMT markers [51].

Besides downregulation of E-cadherin expression, EMT can be stimulated by the proteolysis of E-cadherin extracellular domain. In this context, the shedding of the E-cadherin ectodomain by several proteases including MMP-3, MMP-7, MMP-9, and MMP-14 has been reported [52–54]. Furthermore, breast cancer cells that express the osteoblast differentiation transcription factor known as RUNX2 are able to overexpress MMP-2 that in turn sheds the N-terminal E-cadherin region releasing an 80-kDa soluble E-cadherin (sE-Cad) fragment [55]. These cells also express MMP-11, MMP-12, and MMP-16, but their role in E-cadherin processing is not clear. The sE-Cad fragment is able to bind to the human EGFR also known as HER or ErbB. This binding may be stronger than the one with EGF [56]. Moreover, sE-Cad is able to form complexes with the four EGFRs favoring cancer progression by the activation of the MAPK/ERK and the PKI3/mTOR/Akt signaling pathways [56]. Likewise, the exposure to culture medium from breast cancer cells enriched with sE-cad caused an increase in the synthesis of MMP-2, MMP-9, and MMP-14, as well as in the invasion capacity of human bronchial epithelial cells [57]. Furthermore, sE-Cad can disrupt adhesive junctions by its binding to full-length E-cadherin extracellular domain [58].

On the other hand, the E-cadherin intracellular domain forms a complex with β-catenin attached to the cell membrane thus preventing its transcription activities. When E-cadherin is cleaved, it releases β-catenin to the cytosol in which the

canonical Wnt signaling avoids its degradation with its subsequent accumulation and nucleus translocation [59]. Then, β-catenin forms a complex with the T-cell factor/lymphoid enhancer factor 1 (TXF/LEF1) inducing the synthesis of Twist, Snail, Slug, and c-*myc* that favors EMT and cancer progression, as well as MMP-3, MMP-7, MMP-14, and MMP-26 expression [60]. Interestingly, MMP-3 plays a role in Wnt pathway regulation through its interaction with Wnt3a favoring β-catenin nucleus translocation and transcription functions [60]. Regarding the non-canonical Wnt pathway, expressions of the Wnt5a ligand and its receptor Ror2 are induced by Snail; this complex regulates the synthesis of MMP-2 and MMP-13 in epidermoid carcinoma and osteosarcoma cells, respectively [61]. Likewise, Wnt5a is upregulated in human LNCaP prostate cancer cells that overexpress MMP-14 [54]. Moreover, suppression of Wnt5a synthesis using three different siRNAs inhibits cell migration capacity provoked by MMP-14 expression. In spite of these results, there are controversies about Wnt5a's role in cancer progression. These differences are due to the existence of two Wnt5a isoforms, Wnt5a-long (-L) and the truncated isoform Wnt5a-short (-S) [62]. Wnt5a-S upregulation promotes cell proliferation while Wnt5a-L has opposite effects in neuroblastoma and breast and cervical cancer cells that seem to grow independently of the canonical pathway [62]. Furthermore, Wnt5a-S knockdown decreases cell proliferation and induces apoptosis with an increase in FAS ligand (FASLG) expression and with downregulation of the tumor necrosis factor receptor superfamily member 11 b (TNFRSF11B) in HCT116 colon cancer cells [63].

EMT provides neoplastic cells with mesenchymal characteristics that allow them to invade the surrounding tissue. For example, experiments done in HT29 colon adenocarcinoma cells that overexpress Snail show that the latter drives cathepsin B and MMP-2 to cell membranes increasing MMP-2 pericellular activity [22]. Moreover, EMT and MMPs are involved in the acquisition of stem cell characteristics. In this context, SCC9 human oral squamous cell carcinoma cells that overexpress MMP-14 have a fibroblast-like morphology and an increase in ZEB1, ZEB2, Twist, N-cadherin, fibronectin, and vimentin expression [64]. These cells also have self-renewal, apoptosis, and neomycin resistance. A summary of MMPs' effects on EMT factors and the impact on some molecules involved in EMT is presented in Table 5.2.

5.5 The Role of MMPs in the Evolution of Neoplastic Cells

TME metabolic conditions drive the evolution of tumor cells provoking the presence of a great diversity of cancer cells with variations in differentiation grade, metastatic abilities, cell biomarkers, and therapeutic response. There are several theories that try to explain neoplastic cell heterogeneous population in solid tumors: (1) the hierarchical or cancer stem cell (CSC) theory, (2) the stochastic or clonal model, (3) the CSC dynamic

Table 5.2 MMPs' interactions with EMT

MMP	Effect on EMT	EMT	Effect on MMPs
MMP-2, MMP-3, MMP-7, MMP-9, MMP-14	E-cadherin cutoff	β-catenin	↑ MMP-3, MMP-7, MMP-14, MMP-26
MMP-3	β-catenin nucleus translocation	sE-Cad	↑ MMP-2, MMP-9, MMP-14
MMP-3	↑ Snail ↓ E-cadherin	Snail	↑ MMP-2, MMP-9, MMP-13
MMP-9	↑ Snail	Wnt5a	↑ MMP-14
MMP-14	↑ Slug, Snail, Twist, ZEB1, ZEB2	ZEB1	↑ MMP-2

EMT epithelial-mesenchymal transition, *MMP* matrix metalloproteinase, *sE-Cad* soluble E-cadherin

scheme, (4) the horizontal gene transfer mechanism, and (5) the cell fusion model [6]. TME metabolic characteristics have influence on neoplastic cell evolution. For example, glioma cells isolated from human brain tumors, grown in low pH (6.5), expressed the glioma stem cell (GSC) markers Oct4, Nanog, and Oli2, developed the ability to establish neurosphere-like structures in vitro, and increased their tumor formation capacity in an in vivo experimental model [65]. Interestingly, the exposure of glioma cells to acidic stress also increased the expression of vascular endothelial growth factor (VEGF), an angiogenic factor, and of HIF-2α in normoxic conditions. The expression of HIF-2α was associated with the maintenance of GSCs [65]. Moreover, prostate cancer cells (PC3 cell line) cultured in acidic medium (pH 6.5) acquired CSC-stemness markers, such as CD133, CD44, Oct4, and Klf4, increased their cell viability, enhanced their capacity to form cell colonies, and raised MMP-9 and VEGF synthesis and secretion [66].

On the other hand, MMPs play a role in the acquisition and regulation of CSC functions. For example, it has been demonstrated that MMP-3 is involved in Wnt pathway control in mammary stem cells (MaSCs) [67]. In general terms, the canonical Wnt signaling favors stem cell characteristics while the noncanonical Wnt route controls cell proliferation. MMP-3 hemopexin domain and its hinge region bind to the noncanonical ligand Wnt5b co-receptor site. Moreover, MMP-3 is able to cleave this Wnt5b region interfering with noncanonical Wnt signaling, favoring the canonical Wnt pathway, and therefore promoting MaSC proliferation and differentiation to CSCs [67].

Likewise, MMP-7 has a role in the transdifferentiation from acinar to ductal cells that apparently have progenitor cell-like properties contributing to the initial metaplastic lesions in pancreatic ductal adenocarcinoma (PDA) [68]. In vitro experiments demonstrated that MMP-7 activates the Notch pathway producing a nestin-positive intermediate followed by the adeno-ductal metaplasia that in turn gives rise to the metaplastic duct lesion associated with PDA [68].

Similarly, MMP-9 overexpression was detected in hematopoietic stem cells (HSCs) from the bone marrow after 5-fluorouracil (5-FU) treatment [69]. This MMP promotes HSC differentiation and migration due to the release of soluble kit ligand (sKitL) from stromal cell membrane that binds to KitL receptor (c-Kit) in HSCs [69]. Moreover, myelosuppression induces stromal cell-derived factor-1 (SDF-1) expression in stromal and HSC cells and is responsible for MMP-9 synthesis [69].

Similarly, MMP-10 expression was observed in bronchio-alveolar stem cells (BASCs) that contain a driving Kras mutation (CMT167, a mouse cell line) [70]. These cells grow as non-adherent oncospheres expressing stem cell markers such as CD133, Hey1, Hey2, Aldh1, Notch23, Notch4, and Nanog that get lost when MMP-10 is downregulated. Comparable results were observed in MMP-$10^{-/-}$ Lewis lung carcinoma (LLC) oncosphere cultures [70]. Besides MMP-10's role in the regulation of stem cell gene expression and therefore in their maintenance, MMP-10 favors other stem cell functions such as colony expansion and tumor-initiating activity.

Likewise, MMP-14 plays a role in the regulation of HSC differentiation and maintenance through the binding to FIH-1 [71, 72]. This interaction releases HIF-1α allowing the expression of genes such as SDF-1, KitL, interleukin-7 (IL-7), and erythropoietin (Epo) involved in HSC development. Additionally, SDF-1 also maintains HSCs at the bone marrow through its binding to CXCR4 [73]. But the SDF-1-CXCR4 link can be disturbed by MMPs. For example, MMP-2 hemopexin C region binds to SDF-1 to cleave and release a tetrapeptide from the N-terminal domain of SDF-1 blocking its binding to CXCR4 and thus promoting HSC migration [74]. Moreover, a mass spectrometry assay demonstrated that MMP-1, MMP-3, MMP-9, MMP-3, and MMP-14 cleave SDF-1 in the same site that MMP-2 does [74]. Likewise, MMP-8 from neutrophil granulocytes also promotes HSC mobilization by the disruption of SDF-1 N-terminal region, but the cleavage site is different from the other MMPs since MMP-8 releases a tripeptide from the N-domain [75]. It is important to mention that

SDF-1's first two amino acids are involved in cell migration, whereas the next six amino acids participate in SDF-1 binding to CXCR4 [75].

5.6 MMPs and Non-neoplastic Cells from the TME

Besides a great cancer cell heterogeneity, TME is constituted by blood endothelial cells (ECs), lymphatic endothelial cells (LECs), bone marrow-derived cells, pericytes, smooth muscle cells, adipocytes, neutrophils, tumor-associated macrophages (TAMs), neuroendocrine cells, lymphocytes, myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs), and their interactions allow cancer progression. Only those cells with activities regulated directly or indirectly by MMPs and cells that express MMPs in the TME and have a role in cancer progression are discussed in this chapter.

5.6.1 Immune Response Cells

TME comprises cells that participate in the immune response (Fig. 5.3). Therefore, tumor cells acquire molecular mechanisms to evade immune surveillance that includes MMPs' participation (Fig. 5.3). For example, cervical cancer cells decrease T-cell development by the secretion of MMP-2 and MMP-9 that in turn cleave the interleukin-2 receptor α (IL-2Rα) from the membrane of T cells blocking their proliferation [76]. Likewise, tumor cells express on their membrane ligands that join the natural killer group 2D (NKG2D) receptors from the immune cytotoxic cells such as $CD8^+$ T lymphocytes and natural killer (NK) cells [77]. The binding of these tumor ligands activates the immune response. There are three different types of NKG2D ligands: the UL16 binding proteins (ULBP), the major histocompatibility complex (MHC) class I-related chain molecules A (MICA), and MICB [77]. The expression of these tumor ligands might be regulated by the TME; for instance, hypoxia downregulates the expression of MICA through HIF-1α contributing to the escape of tumor cells from the immune surveillance [77]. Moreover, the proteolytic cleavage of MICA and MICB by MMPs produces the release of ligands that in turn compete for the immune cell receptors avoiding cytotoxic effects particularly from NK cells. In this context, MMP-2 is able to cut off MICA from renal carcinoma cells, while MMP-9 cuts it from osteosarcoma cells [78, 79]. Moreover, MMP-14 is involved in MICA shedding from prostate and breast cancer cells [80]. It is important to note that MMP-14's role in MICA cleavage is independent of MMP-2 and MMP-13 activation. Interestingly, the MMPs from CAFs may also participate in these evasion mechanisms. Melanoma cells cultured with conditioned medium (CM) rich in active MMPs from melanoma-associated fibroblasts decrease their susceptibility to NK-related cytotoxic effects, increase soluble MICA and MICB fragments released to the cultured medium, and reduce MICA and MICB expression in their cell membranes [81]. These effects were reversed by the MMPs' inhibitor GM6001. The CM comprised several MMPs including MMP-2, MMP-9, and MMP-14, but it was not clear which MMP was responsible for NKG2D shedding.

Tumors also suppress the immune response through the secretion and activation of transforming growth factor-β (TGFβ) located in the ECM or released by other cells. TGFβ is synthesized as a pro-protein in which the pro-peptide has a furin recognition sequence that is cleaved by furin, but other proteases such as thrombin, neutrophil elastase, plasmin, and MMPs can also disturb it [82]. In this context, MMP-2 and MMP-9 can activate TGFβ2 and TGFβ3 with little impact on TGFβ1 [82]. In contrast, MMP-13 seems to have more effect on TGFβ1. Likewise, MMP-14 activates TGFβ when it is presented by αvβ8 integrin on cells' surface [82]. TGFβ activation suppresses lymphocyte T proliferation and differentiation and interferes with antigen presentation [83]. Moreover, active TGFβ from platelets downregulates tumor NKGD2 decreasing NK cell anti-tumor responsiveness [84].

5.6.2 Tumor-Associated Macrophages (TAMs)

On the other hand, TAMs are the largest innate immune cell population in the TME. TAMs originate from monocytes that are attracted from blood circulation to the tumor by cytokines such as SDF-1 also called C-X-C motif chemokine 12 (CXCL12), chemokine C-C motif ligand 2 (CCL2), RANTES (**R**egulated on **A**ctivation, **N**ormal **T** cell **E**xpressed and **S**ecreted, also known as CCL5), monocyte colony-stimulating factor (M-CSF), VEGF, and TGFβ, all of which are secreted by cancer and stromal cells [85]. In this context, there is a close interaction among TAMs and neoplastic cells in breast cancer [86]. Tumor cells release the M-CSF while TAMs express the CSF receptor (CSFR), and TAMs produce EFG, whereas neoplastic cells have the corresponding receptor. TAM polarization depends on TME conditions. Differentiation to classically activated M1 macrophages in response to lipopolysaccharide (LPS) and interferon-γ (IFN-γ) is observed in normoxic areas, and alternatively activated M2 cells emerge under the influence of IL-4 and IL-13 mainly in hypoxic zones [85]. M1 macrophages have cytotoxic phenotype since they secrete IL-6, IL-12, reactive nitrogen intermediates, tumor necrosis factor-α (TNFα), and ROS, while M2 macrophages participate in ECM remodeling through the release of MMPs particularly the M2c phenotype and promote angiogenesis, neoplastic cell stemness, and chemotherapy resistance [85, 86]. Since M2 macrophages are associated with cancer progression, in the present chapter, we focus on this type of TAMs. In this context, monocytes' differentiation into TAM M2 is driven by neoplastic cells. However, TAMs have influence on tumor cells' behavior. Co-cultures of MKNI gastric carcinoma cells with monocyte cells induce a morphologic change together with a rise in the expression of α5 integrin in their cell membranes and high levels of secreted MMP-9 and fibronectin with an increase in their invasion capacity [87]. These monocytes' effects on cancer cells are dependent, at least in part, on TNFα. Similarly, M2 cells from renal cell carcinoma synthesize high levels of IL-1β that induce tumor MMP-1, MMP-3, MMP-10, and MMP-14 expression in patients with advanced stages of the disease [88]. Moreover, M2-like macrophages are able to stimulate the synthesis of MMP-2 and MMP-9 in SW480 human colon cancer cells with the induction of EMT and the increase in their invasiveness ability [89]. Likewise, TAM-like cells secrete high levels of MMP-9 [87]. In fact, MMP-9 has been considered as a marker of M2 macrophages and therefore can be used to predict breast cancer patients' outcome [90]. Furthermore, a co-localization of MMP-9 and MMP-11 in M2 macrophages was demonstrated in tissue samples from cutaneous squamous cell carcinoma [91]. MMP-1 and MMP-12 were also observed in TAMs together with deposits of periostin, a non-structural ECM protein, at the peripheral zones of dermatofibrosarcoma protuberans (DFSP) [92]. In this regard, treatment of $CD163^+$-monocyte-derived macrophages with periostin and IL-4 stimulated the synthesis of MMP-1 and MMP-12, while periostin alone only augmented MMP-12 expression [92]. Interactions among neoplastic cells and TAMs through MMPs are outlined in Fig. 5.3.

5.6.3 Tumor-Associated Neutrophils (TANs)

Even though circulating neutrophils have been considered as cells with a short lifetime and with cytotoxic effects, they are part of the TME and their presence has been correlated with a poor prognosis. Moreover, cytokines, such as IL-1, prolonged their life and contributed to neutrophil polarization allowing them to participate in cancer progression [93]. Like TAMs, there are two different types of tumor-associated neutrophils (TANs) with different functional characteristics. Monocytes exposed to IFNβ differentiate into the N1 type while stimulation by TGFβ promotes N2 polarization [94]. N1 neutrophils are present in cancer early stages, have cytotoxic and antitumoral functions, and are able to attract $CD4^+$ and $CD8^+$ lymphocytes to tumor lesions. In contrast, N2 cells possess immunosuppressive

characteristics since, under the influence of tumor IL-8, they can suppress $CD8^+$ cytotoxic cells by the secretion of arginase 1, are able to recruit forkhead box P3 ($FoxP3^+$) cells also known as Tregs cells that induce tumor growth and suppress anti-tumor response, and promote angiogenesis [94, 95]. TAN granules contain several proteases such as cathepsin G, neutrophil elastase (NE), and MMPs that contribute to the ECM modification as well as to tumor growth and metastatic capacity (Fig. 5.3) [96]. For instance, NE and MMP-9 are involved in BM disruption favoring the release of VEGF that is a potent pro-angiogenic factor. In fact, TANs are one of the major sources of MMP-9 in TME. For example, MMP-9 was increased in TANs but not in TAMs in NSCLC tissue [97]. Further, it has been observed that MMP-9 was augmented in TANs compared to TAMs at the leading edge of the tumor in a model of murine pancreatic adenocarcinoma [98]. Interestingly, the MMP-9 released by TANs is a TIMP-1-free pro-MMP-9 while the MMP-9 secreted by other cells is in complex with TIMP-1 (see above) [99]. Additionally, only the TIMP-1-free pro-MMP-9 from TANs is able to induce angiogenesis in in vivo models. Furthermore, this TIMP-1-free pro-MMP-9 is activated through MMP-3 in collagen on plants while it undergoes natural activation in in vivo models. Likewise, MMP-9 for its pro-angiogenic function requires both the catalytic and the hemopexin domains [99]. TAN MMP-9 also promotes basic fibroblast growth factor-2 (bFGF2) activation that has pro-angiogenic properties, too [99]. It is important to note that TAMs also produce TIMP-1-free pro-MMP-9 through shutting down TIMP-1 expression when they are polarized to M2-like macrophages [100]. This TAM MMP-9 also induces angiogenesis; however, M2 macrophages release lower quantities of pro-MMP-9 in comparison to TANs that are able to rapidly secrete great amounts of pro-MMP-9 stored in their granules [100]. Additionally, MMP-9 enzymatic activity may be protected and enhanced by the neutrophil gelatinase-associated lipocalin (NGAL) released by neutrophils and cancer cells [101]. MMP-9 and NGAL form a complex that has been associated with an increase in angiogenesis and metastatic potential and therefore is considered as a bad prognosis marker for cancer outcome [102]. Likewise, TAN MMP-9 stimulates tumor cell proliferation and reduces apoptosis [103, 104].

Likewise, tumor-infiltrating neutrophils recruited and polarized to TANs N2 by bladder cancer cells are able to increase neoplastic cell invasion capacity via upregulation of androgen receptor (AR) expression that in turn enhances MMP-13 synthesis [105].

On the other hand, neoplastic cells promote neutrophil extracellular trap (NET) production by neutrophils [106]. NETs are neutrophil DNA decorated with neutrophil peptides. In the case of cancer, among NETs' components are cathepsin G, neutrophil elastase, and MMP-9. The functions of NETs in cancer are not well defined but probably they favor tumor proliferation, resistance to apoptosis, detachment from the primary tumor, and induction of angiogenesis [106].

5.6.4 Mast Cells

Mast cells (MCs) are recruited from blood circulation to the TME by CCL15 or stem cell factor (SCF) secreted by neoplastic cells. Likewise, TGFβ and cytokines such as IL-3, IL-4, IL-9, IL-10, IL-33, and SDF-1 contribute to MC growth and maintenance [107]. Once in the tumor, the TME might regulate MC behavior, and as TAMs and TANs, MCs differentiate into two types: MCs that contain tryptase (T-MCs) and the MCs that secrete tryptase and chymase (TC-MCs) [108]. Moreover, T-MCs can evolve to TC-MCs depending on the TME conditions such as the presence of IL-4, IL-6, nerve growth factor (NGF), and SCF [108]. Even though it was possible to identify different MC types, it is not clear which one has anti-tumor or pro-cancer characteristics.

Among their anti-cancer actions, MCs are able to engage cells from the innate immune system to establish an anti-tumor immune response, tumor growth suppression by IL-1, IL-6 and TNFα secretion, apoptosis promotion, and inhi-

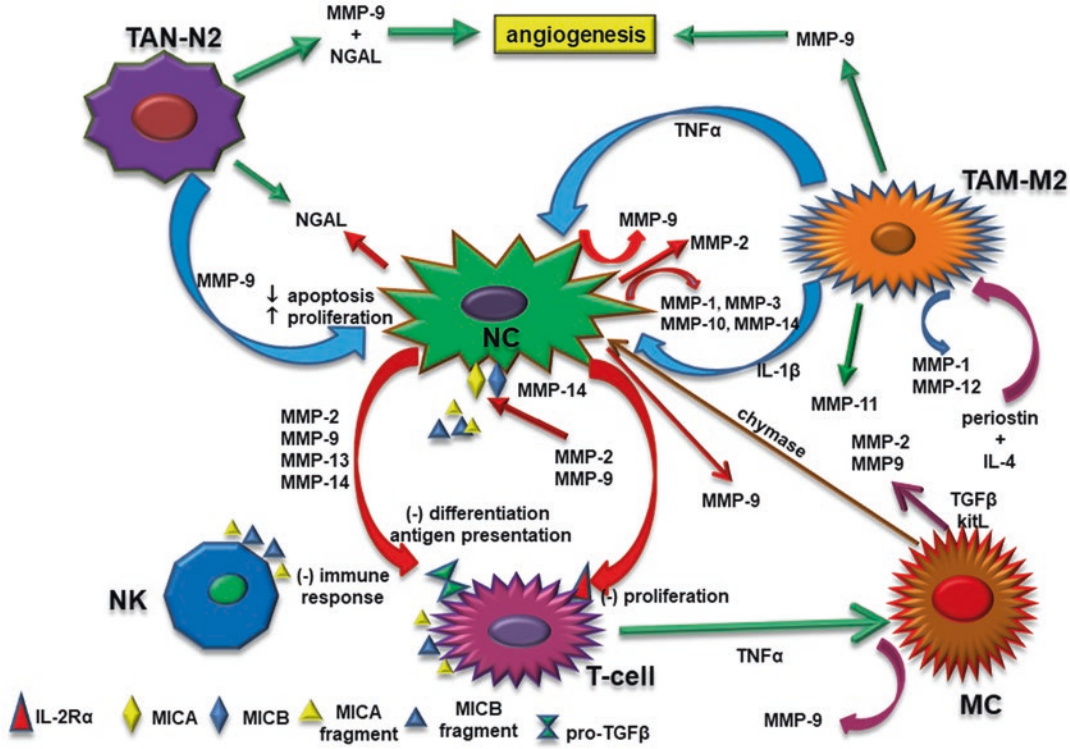

Fig. 5.3 Crosstalk among neoplastic and inflammatory cells. Neoplastic cell (NC) interferes with the immune response through the release of MMP-2 and MMP-9 that inhibit T-cell proliferation. These MMPs and MMP-14 cut off MICA and MICB from tumor cell membranes to evade the immune response. MICA and MICB fragments bind to immune cell receptors contributing to a decrease in immune surveillance. Tumor cell MMP-2, MMP-9, MMP-13, and MMP-14 activate TGFβ blocking T-cell differentiation and antigen presentation. TAMs release TNFα that increases neoplastic cell MMP-9 expression while IL-1β induces MMP-1, MMP-3, MMP-10, and MMP-14. Periostin upregulates MMP-12 synthesis while periostin and IL-4 promote the expression of MMP- 1 and MMP-12 from TAMs. TAMs also produce MMP-9 and MMP-11. TANs release MMP-9 that favors angiogenesis. MMP-9 pro-angiogenic function is protected by NGAL synthesized by TANs and neoplastic cells. MMP-9 blocks tumor cell apoptosis and increases cell proliferation. MC MMP-9 expression is induced by activated T cells through TNFα. Likewise, MMP-2 and MMP-9 are stimulated by TGFβ and KitL. MC chymase promotes MMP-9 expression from NC. IL interleukin, IL-2Rα interleukin-2 receptor α, KitL kit ligand, MC mast cell, MMP matrix metalloproteinase, MICA MHC class I-related chain molecules A, NC neoplastic cell, NGAL neutrophil gelatinase-associated lipocalin, NK natural killer, TAN tumor-associated neutrophil, TAM tumor-associated macrophage, TGFβ transforming growth factor-β

bition of cell migration [109]. Conversely, MCs are able to induce cancer progression by increasing tumor cell growth and ECM disruption to facilitate neoplastic cell mobilization and to promote angiogenesis [109]. Furthermore, MCs can provoke an immunosuppressive response to NK and T cells by releasing adenosine in the TME [107]. Likewise, cellular immunity is regulated by MC secretion of TNFα, IL-10, and histamine [109]. Interestingly, an intratumoral location of MCs is associated with a good prognostic which indicates that these MCs have anti-tumoral properties. In contrast, MCs that promote cancer progression are located at the tumor invasive front next to the neovascularization areas [109].

On the other hand, MCs synthesize and activate MMPs (Fig. 5.3). For instance, MMP-9 is produced by contact among MCs and activated lymphocytes through TNFα [110]. Moreover, MC MMP-2 as well as MMP-9 expression might

be regulated by TGFβ and KitL [111]. Likewise, MMP-9 is expressed in MCs in well-differentiated prostate adenocarcinomas but not in MCs from poorly differentiated tumors that show an enhanced MMP-9 location in cancer cells [112]. These results suggest that, through MMP-9, MCs are involved in prostate cancer development. Similarly, MCs also have the capacity to express MMP-1 although the specific function of this MC MMP in cancer progression has not been examined [113]. In this context, a correlation among MC density and MMP-9 has been reported in multiple melanoma (MM) patients with osteolytic disease [114]. The role of MCs besides angiogenesis promotion is the increase of bone re-absorption in MM progression. Therefore, although MC MMP-1's presence in this pathology has not been described, it is possible that both MMP-9 and MMP-1 from MCs are involved in angiogenesis and bone re-absorption, respectively.

Likewise, MC chymase can stimulate MMP-9 expression in A549 lung adenocarcinoma and in H520 squamous lung carcinoma cells [115]. Moreover, chymase also participates in EMT induction by shedding E-cadherin from tumor cell surface [115]. Furthermore, MC tryptase plays a role in the activation of MMPs and plasminogen activator (PA) [116].

5.6.5 Cancer-Associated Fibroblasts (CAFs)

The most abundant TME non-neoplastic cells are CAFs. Cell origin of CAFs is variable since they can derive from local fibroblasts, senescent fibroblasts, smooth muscle cells, pericytes, ECs, epithelial cells, adipocytes, hematopoietic stem cells, and bone marrow-derived mesenchymal stem cells (BMDMCs) [117, 118]. Neoplastic cells are responsible for the recruitment and transformation of cells into CAFs through the secretion of different growth factors and cytokines [119]. Once cells arrive to the TME, they might transform into CAFs through different processes, for instance, (1) epithelial cells via EMT, (2) bmMSCs via the osteospondin-myeloid zinc finger 1 (OPN-MZF1)-TGFβ1 pathway, (3) resident fibroblasts through mesenchymal-mesenchymal transition (MMT), and (4) ECs via endothelial-mesenchymal transition (endMT) [118, 120]. These events are also induced by cancer cells. Interestingly, MMPs such as MMP-1, MMP-2, and MMP-14 from the neoplastic cells promote fibroblast MMT [119]. The maintenance of CAFs is mediated by paracrine signals from neoplastic cells and, as it happens with them, CAFs may get independence from the TME by generating autocrine signals for survival [121, 122]. Activated CAFs are able to influence TME cells including cancer cells through the synthesis and secretion of growth factors, cytokines, and ECM structural and non-structural proteins [123]. Likewise, interactions of CAFs with the ECM allow them to sense its stiffness and re-organize its molecules favoring neoplastic cell migration [124]. In this context, CAFs produce proteolytic enzymes such as lysyl oxidases and MMPs. It has been reported that active CAFs are able to express MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13, MMP-14, and MMP-19 (Fig. 5.4) [125]. These enzymes allow ECM remodeling through degradation of the BM and of some molecules from the interstitial ECM. Furthermore, cancer cells induce MMP synthesis from CAFs. For example, treatment of human mammary primary fibroblasts with CM from breast carcinoma cells elevated MMP-1 expression and favored transdifferentiation to CAFs [126]. Moreover, CAFs from primary breast cancer synthesized more MMP-1 in comparison to normal mammary fibroblasts. Besides its role in ECM degradation, MMP-1 is able to disrupt the protease receptor-1 (PAR-1) in a specific site that allows its activation and generation of PAR-1-dependent Ca^{2+} signals promoting tumor cell migration [127]. Moreover, PAR-1 expression is regulated by TGFβ through Smad3 and Smad4 with an increase of osteoclast differentiation, tumor growth, and angiogenesis in an in vitro model of bone giant tumor cells [128]. TGFβ is activated by several MMPs; therefore, MMPs stimulate PAR-1's functions in a direct and indirect manner in cancer progression [82]. Likewise, CAFs and neoplastic cells at

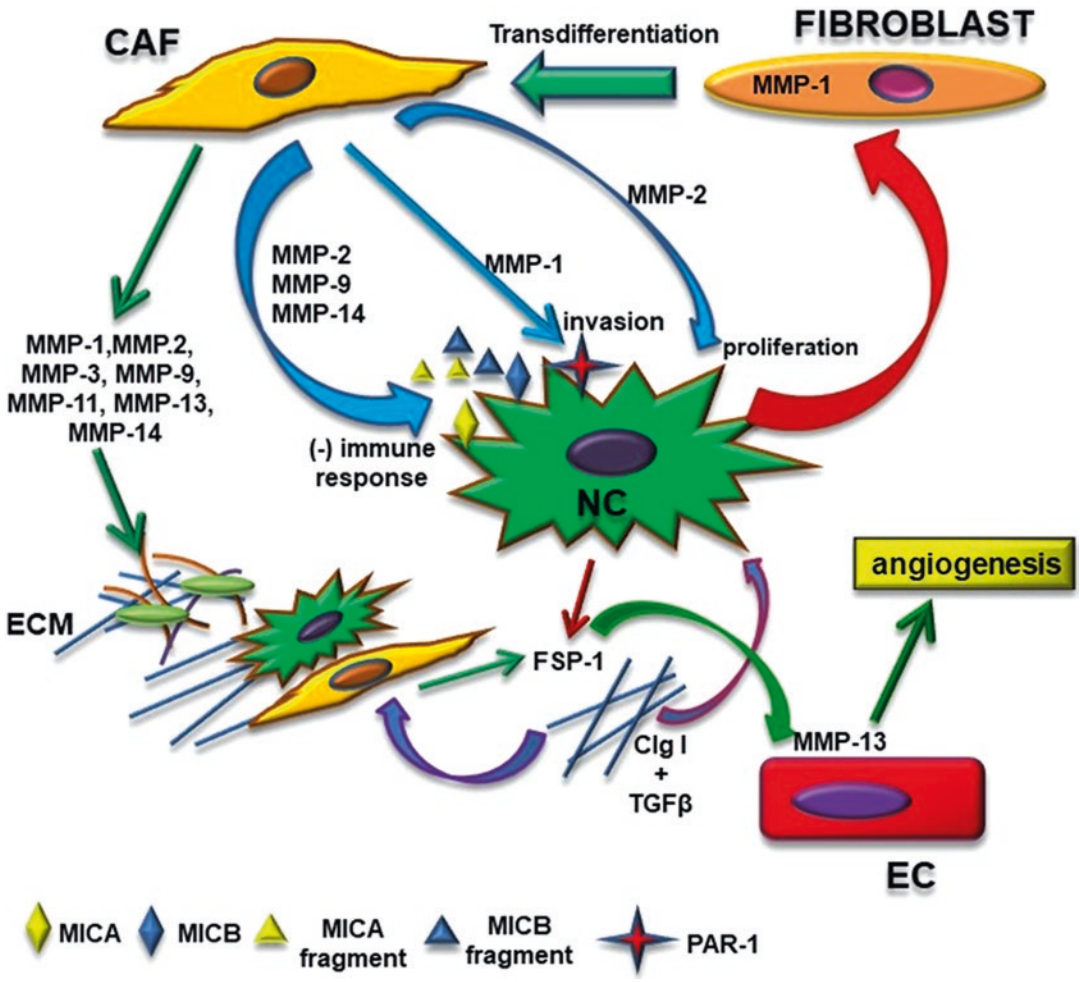

Fig. 5.4 The interactions of CAFs with neoplastic cells. NCs induce normal fibroblast MMP-1 upregulation for their transdifferentiation into CAFs. Disruption of the BM and ECM is driven by several MMPs secreted by CAFs, promoting NC invasion and angiogenesis. Moreover, interstitial MMPs (collagenases) as well as MMP-2 are involved in the creation of paths for NC and CAF mobilization. Likewise, ECM clg I and TGFβ stimulate FSP-1 expression from CAFs and NCs. FSP-1 favors MMP-13 EC expression that induces cell migration during angiogenesis. MMP-2 and MMP-1 secreted by CAFs promote NC proliferation and invasion, respectively. Furthermore, CAFs participate in NC escape from the immune response through MMP-2, MMP-9, and MMP-14 MICA and MICB cutoff. CAFs cancer-associated fibroblasts, clg I type I collagen, EC endothelial cell, ECM extracellular matrix, FSP-1 fibroblast-secreted protein-1, MICA MHC class I-related chain molecules A, MICB MHC class I-related chain molecules B, MMP matrix metalloproteinase, NC neoplastic cell, PAR-1 protease-activated receptor-1, TGFβ transforming growth factor-β

the invasive front move together across tracks formed by CAFs through ECM contraction in which rhoA-dependent kinase (ROCK) is involved and with the ECM remodeling by MMPs and the deposition of ECM components such as tenascin-C and fibronectin [129, 130].

Activated CAFs can also express MMP-2. Immunohistochemical analysis revealed a co-localization of MMP-2 and α-smooth muscle actin (α-SMA) and vimentin, both CAF markers, at the perimeter of lung metastasis [131]. Moreover, a decrease in tumor lesions was observed in MMP-$2^{-/-}$ mice in comparison with

wild animals in which tumor cells were injected. The role of MMP-2 in tumor proliferation was tested when MMP-2$^{-/-}$ fibroblasts were cultured in direct contact with tumor cells resulting in a decrease in the spheroid tumor growth [131].

CAFs are able to express MMP-13 and to induce its synthesis by other cells. This MMP is important in cancer progression since MMP-13 increases the invasive growth of cancer cells and favors angiogenesis through the release of VEGF from the ECM [132]. Similarly, MMP-13 activates TGFβ that, with type I collagen, stimulates the expression of fibroblast-secreted protein-1 (FSP-1) also known as S100A4 in CAFs and neoplastic cells [133]. FSP-1 can promote MMP-13 expression and its enzymatic activity in ECs favoring their migration during angiogenesis [134]. Furthermore, FSP-1 also activates plasminogen that has pro-angiogenic properties and induces EMT in cancer cells [133]. Likewise, TGFβ activation upregulates CXCR4, the SDF-1 receptor. The interaction of SDF-1 and CXCR4 increases neoplastic cell proliferation. Moreover, SDF-1 acts as a chemoattractant for those cells that express CXCR4 favoring the recruitment of EC precursors and the migration of cancer cells to LN and to other organs rich in SDF-1 [133]. Paradoxically, MMP-13 cleaves SDF-1 causing its inactivation [133]. In this context, more studies are needed to clarify the role and the molecular mechanism involved in SDF-1 regulation by MMP-13.

Another TME molecule that participates in the regulation of MMPs' expression in CAFs is the plasmin inhibitor TFPI-2 (tissue factor pathway inhibitor-2). Assays done with lung fibroblasts cultured with CM from TFPI-2 silenced NCI-H460 cells (an NSCLC cell line) showed an increase in MMP-1, MMP-3, and MMP-7 synthesis [135]. Likewise, fibroblasts that overexpress phosphoglycerate kinase-1 (PGK1) have high levels of SDF-1, vimentin, and α-SMA and an increase in their growth index. Moreover, these fibroblasts express high levels of MMP-2 and MMP-3 that participate in prostate cancer cell invasion [136].

5.6.6 Adipocytes

Adipocytes are a major component of the TME. They secrete cytokines, pro-angiogenic factors, and adipokines that favor tumorigenesis and cancer progression. Moreover, they are a source of fatty acids that supply the energetic requirements of neoplastic cells [137]. Adipocytes have a different distribution pattern throughout the tumoral tissue [138]. For instance, there are more adipocytes with a fibroblast-like cell morphology and CAFs than mature adipocytes surrounding neoplastic cells at the tumor invasive front. In this context, adipocytes from this region under the influence of neoplastic cells transdifferentiate into adipocyte-derived fibroblasts (ADFs) via the activation of the Wnt/β-catenin pathway (Wnt canonical pathway) [139]. Interestingly, MMP-7 is expressed by ADFs probably as a consequence of the activation of the Wnt/β-catenin pathway (Fig. 5.5) [139]. It is important to note that other MMPs regulated through this mechanism at the transcriptional level (MMP-3, MMP-14, and MMP-26) can also be expressed by ADFs (see above). Likewise, ADFs acquire CAF markers such as FSP-1, type I collagen, and fibronectin but not α-SMA during their differentiation [140]. ADFs in turn develop into CAFs, although the specific mechanism is not clear.

Adipocytes also influence tumor cells by promoting CSC characteristics. In this context, breast cancer cells that express the estrogen receptor (ER^{+}) co-cultured with human adipocytes (SGBS cell line) in hypoxic conditions demonstrated an increase of the EMT transcription factors FOXC2 and TWIST together with an increase of N-cadherin expression and a decrease in E-cadherin synthesis [141]. Besides, adipocytes had an increase in TGFβ, lectin-type oxidase LDL receptor-1 (LOX1), and HIF-1α expression.

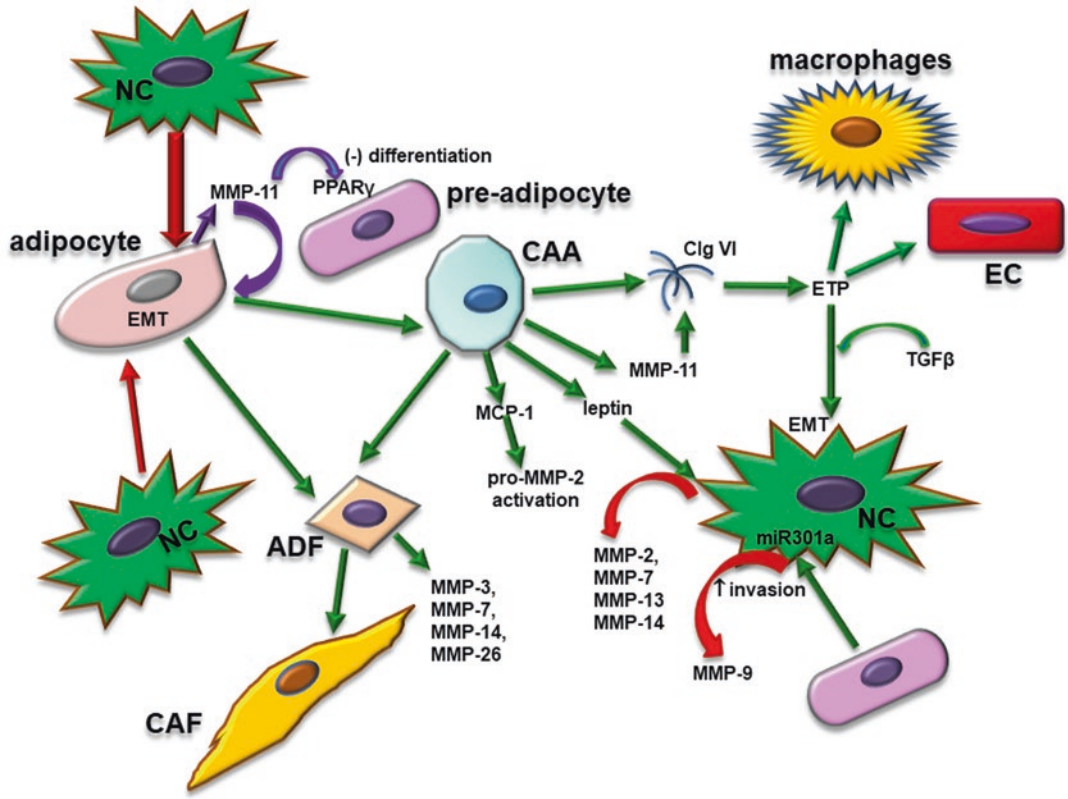

Fig. 5.5 TME different adipocyte types' role in cancer progression. NCs induce EMT in adipocytes favoring differentiation into ADFs that can synthesize MMP-3, MMP-7, MMP-14, and MMP-26. NCs also promote adipocyte MMP-11 expression that in turn downregulates PPARγ blocking pre-adipocyte differentiation. MMP-11 provokes metabolic changes in adipocytes transforming them into CAAs. CAAs produce MMP-11 and clg VI. Moreover, MMP-11 degrades clg VI releasing a fragment called ETP. ETP and TGFβ promote EMT and recruit macrophages and ECs to TME. CAAs express MCP-1 that activates pro-MMP-2. Leptin released by CAAs promotes MMP-2, MMP-7, MMP-13, and MMP-14 upregulation in NCs. Pre-adipocyte can induce MMP-9 expression and NC invasion capacity through miR301a regulation. ADF adipocyte-derived fibroblast, CAF cancer-associated fibroblast, CAA cancer-associated adipocyte, clg VI type VI collagen, EC endothelial cell, EMT epithelial-mesenchymal transition, ETP endotrophin, MCP-1 monocyte chemotactic protein-1, MMP matrix metalloproteinase, NC neoplastic cell, PPARγ peroxisome proliferator-activated receptor-γ, TGFβ transforming growth factor-β

On the other hand, cancer cells in close proximity to adipocytes induce them to express MMP-11 at the peritumoral area (Fig. 5.5) [142]. MMP-11 inhibits the differentiation of pre-adipocytes to mature adipocytes and their maintenance by downregulation of peroxisome proliferator-activated receptor-γ (PPARγ) involved in adipogenesis. Moreover, MMP-11 promotes adipocyte lipolysis, decreases the size and number of lipid droplets, and induces changes in adipocyte morphology with a loss of adipocyte markers and a reduction in cell size. These adipocytes are called cancer-associated adipocytes (CAAs) that in turn may dedifferentiate into ADFs (see above) [142].

Activated CAAs secrete adipokines such as TNFα, leptin, hepatic growth factor (HGF) and IL-6, MMP-11, type VI collagen, and fibronectin [140]. In this regard, MMP-11 is able to cut off type VI collagen releasing a C-terminal fragment known as endotrophin (ETP) [143]. ETP induces EMT in tumor cells through its interaction with

the TGFβ and participates in the recruitment of macrophages and ECs [144]. Moreover, ECs migrate and organize vasculature structures more actively in the presence of ETP [144].

Adipocytes also secrete monocyte chemotactic protein-1 (MCP-1) that favors tumor growth, migration, and invasion and participates in pro-MMP-2 activation without increasing its synthesis (Fig. 5.5) [145]. Likewise, pre-adipocytes have effects on the invasion capacities of prostate cancer cells through the induction of miR301a that decreases AR expression with a subsequent TGFβ, Smad3, and MMP-9 upregulation [146].

CAAs also release leptin, a 16-kDa protein that stimulates growth and proliferation of breast cancer cells when adipocytes are in close proximity to neoplastic cells [140]. The effects on cancer cells are due to the leptin receptors (ObRs) [147]. ObRs are expressed in six isoforms, of which the long form called OB-Rb is present in cancer cell membranes. Leptin binding to OB-Rb induces the activation of several pathways such as PI3K/Akt, MAPK/ERK, and JAK/STAT3 involved in tumor cell proliferation and cancer progression. In this context, activation of JAK/STAT3 signaling pathway induces MMP-13 but not MMP-2, MMP-9, or MMP-7 expression in pancreatic cancer cells (Fig. 5.5) [148]. These results were also observed in glioma cells treated with leptin [149]. Moreover, OB-Rb overexpression was observed associated with MMP-13 upregulation and with an increase in lymph node (LN) metastasis in advanced stages of pancreatic cancer [148]. Likewise, ER^+ breast cancer cells co-cultured with adipocyte/stromal stem cells (ASCs) , in which leptin was knocked down, expressed lower levels of MMP-2, IL-6, and serine protease inhibitor E1 (SERPINE1) [150]. Moreover, when breast cancer cells mixed with leptin shRNA ASCs were implanted in the fat pad of mice, tumor growth and metastasis to the liver and lung were decreased in comparison with control animals [150]. Additionally, ASCs synthesize MMP-15 involved in the migration of these cells through the ECM [151].

Leptin promotes activation of c-Jun N-terminal kinase (JNK) and ERK signaling pathways with the induction of MMP-7 expression in ovarian cancer cell lines that express OB-Rb [152]. The induction of MMP-7 synthesis through JNK and ERK pathways was also observed in ovarian cancer cells that expressed mesothelin (MSLN) [153]. This protein induces MMP-7 upregulation via JNK, ERK 1/2, and Akt signaling pathways with an increase in the invasive potential of cancer cells. These findings indicate that leptin and MSLN receptors activate the same molecular mechanisms to promote MMP-7 expression in ovarian tumor cells and probably the synergistic effects of both molecules are involved in ovarian cancer progression. Interestingly, silencing MMP-7 reduces MMP-9 activity but not MMP-9 expression with no effects on MMP-2 [152]. In contrast, MMP-7 was able to induce pro-MMP-9 and pro-MMP-2 activation in other experimental assays [154]. Moreover, these studies showed that MMP-7 dissociated the pro-MMP-2/TIMP-2 complex and activated latent MMP-2 [154].

Leptin also enhances MMP-14 expression and membrane location in gastric cancer cells [155]. MMP-14 location on membrane surface depends on kinesin-like protein 1B (KIF1B) whose expression is also regulated by leptin. As was discussed earlier, leptin stimulates several signaling pathways involved in MMPs' synthesis. In the case of MMP-14, the interaction between leptin and OB-Rb stimulates the Akt pathway for MMP-14 and KIF1B expression [155]. Furthermore, the effect of leptin on MMP-14 synthesis requires the participation of Notch1 signaling. Blocking Notch1 in human extravillous trophoblast cells treated with leptin also reduced Akt phosphorylation and MMP-14 expression [156]. Therefore, the interaction among Notch1 and PI3K/Akt pathway is important to leptin-induced MMP-14 expression.

5.7 Extracellular Vesicles and MMPs

So far, we have reviewed the intricate crosstalk among the different cellular components of the TME including tumor cells in relation with MMPs' participation in cancer evolution. This cellular communication induces the release of factors that stimulate MMPs' synthesis and secretion as well as MMPs' involvement in cellular behavior and ECM disruption, promoting the detachment of neoplastic cells from the primary tumor and their passage through the next steps of the metastatic cascade. Most of these signals between cells are transmitted through extracellular vesicles (EVs) to neighboring cells or to distant sites such as the pre-metastatic niche. EVs are lipid bilayer structures that carry different molecules such as transmembrane proteins, cytosolic proteins, lipids, DNA, microRNAs, and RNA transcripts. There are different types of EVs: microvesicles, exosomes, oncosomes, and ectosomes [157]. EVs release their cargo molecules spontaneously or under some kind of stimuli at the pericellular space, ECM surrounding, into the corporal fluids such as lymph or blood, or into the target cells through membrane-to-membrane interaction, membrane fusion, or EV internalization [158]. Once EVs are in the cells, they may either be degraded with their cargo components, modified and remitted, or disrupted to release EV transported molecules that influence cells' behavior. Transportation of macromolecules in EVs protects them from being degraded and allows them to maintain their integrity and activity. In this context, oncosomes (oncogenic-cargo EVs) are able to transport DNA sequences including KRAS and MYC, transcripts such as EGFRvIII and BRAF, or proteins with a pro-cancer activity [158].

Interestingly, some MMPs have been identified within exosomes. For example, pro-MMP-9 and active MMP-9 in EVs were found in fibrosarcoma and breast cancer cells [157]. Moreover, latent and active forms of MMP-2 and MMP-9 have been observed in ovarian cancer cells [159]. Additionally, heat shock protein-90 (Hsp-90) is transported in exosomes to the pre-metastatic niche where it is able to induce MMP-2 expression [159]. MMP-14 together with β1 integrin is also carried in exosomes in melanoma and fibrosarcoma cells [160]. These exosomes are able to activate pro-MMP-2 and to disrupt gelatin and type I collagen.

5.8 Escape Routes from TME

Besides orchestrating non-neoplastic cells' behavior, tumor cells need to develop strategies to face TME metabolic changes to continue proliferating and to design escape routes to migrate to other tissues. Such is the case of angiogenesis and lymphangiogenesis, processes in which MMPs also have an important role.

5.8.1 Angiogenesis

Angiogenesis consists in the generation of new vessels from preexisting ones under the stimulation of TME conditions. Several angiogenic models have been proposed but the most studied is sprouting [161]. The cells involved in angiogenesis are called tumor endothelial cells (TECs) that may originate from normal ECs, bone marrow-derived progenitor cells, neoplastic cells, and CSCs [6]. TECs respond to different pro-angiogenic factors such as adrenomedullin (AM), EGF, and VEGF [162, 163]. Moreover, TECs produce VEGF in an autocrine manner. Cancer cells as well as other cellular components from the TME also produce pro-angiogenic molecules. For instance, CAFs release TGFβ, SDF-1, and VEGF, and tumor cells produce VEGF, AM, EGF, angiopoietin, and bFGF among others [163]. TECs under the effects of angiogenic factors migrate and participate in the formation of tube structures and stabilization and maturation of the new vessels by the secretion of BM components and pericyte recruitment [164].

Likewise, MMPs are also secreted from tumor and stromal cells contributing to angiogenesis (Fig. 5.6). For instance, MMP-1 increases vascu-

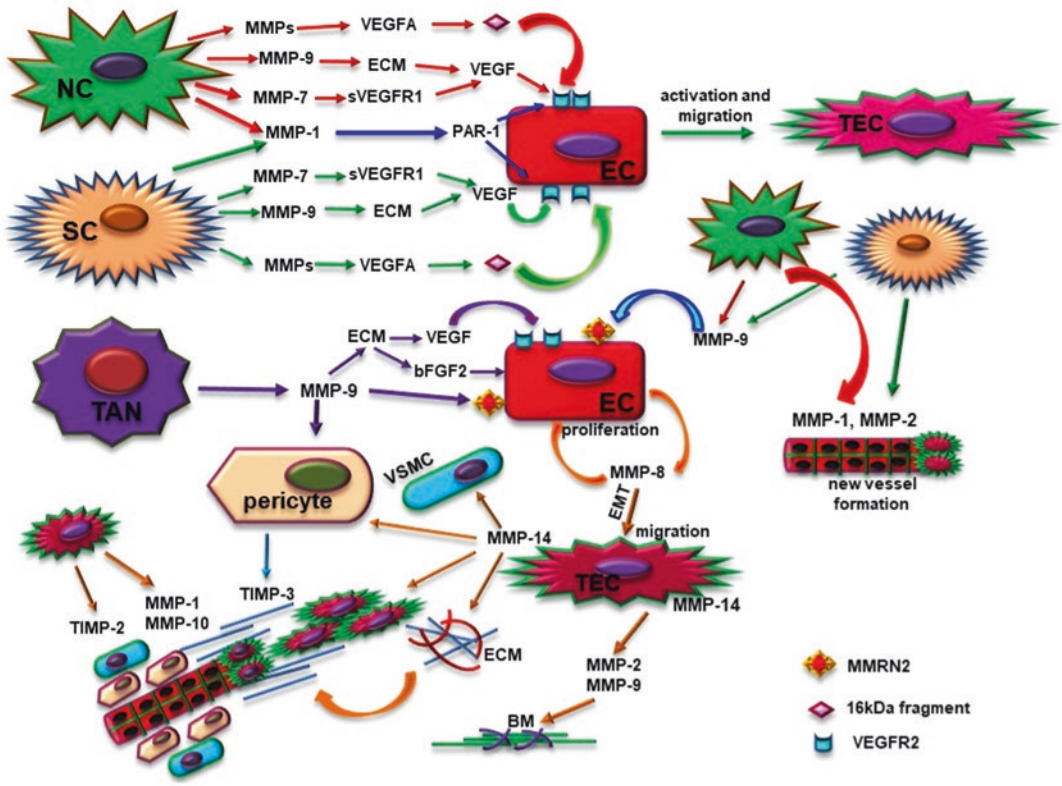

Fig. 5.6 Participation of MMPs in angiogenesis. NCs and SCs produce MMPs that modify EC behavior. MMP-1 disrupts PAR-1 promoting EC expression of VEGFR2. MMP-7 degrades sVEGFR1 to enhance VEGF bioavailability. MMP-9 releases VEGF from the ECM. MMPs such as MMP-3, MMP-7, and MMP-19 cleave VEGFA releasing a 16-kDa fragment that binds to VEGFR2. MMP-1 and MMP-2 participate in tubulogenesis. Likewise, MMP-9 from TANs releases VEGF and bFGF-2 from the ECM, participates in pericyte recruitment, and, with MMP-9 from other cellular sources, degrades MMNR2 from EC membranes. MMP-8 induces EC proliferation and participates in EMT promoting TEC activation and migration. TECs secrete MMP-2 and MMP-9 to degrade BM during cell migration. MMP-14 from TEC membrane activates pro-MMP-2 favoring TEC migration. MMP-14 remodels the ECM to form the vascular guidance tunnels. MMP-14 induces TEC aggregates that migrate and regulate tubulogenesis. MMP-14 favors VSMC transdifferentiation to a mesenchymal phenotype that participates in tube maturation. This MMP also promotes pericyte recruitment. TECs secrete MMP-1 and MMP-10 to limit angiogenesis. Pericytes and TECs release TIMP-3 and TIMP-2, respectively, to avoid the collapse of new vessels. BM basement membrane, bFGF-2 basic fibroblast growth factor-2, EC endothelial cell, ECM extracellular matrix, EMT epithelial-mesenchymal transition, MMP matrix metalloproteinase, MMNR2 multimerin-2, NC neoplastic cell, PAR-1 protease-activated receptor-1, SC stromal cell, sVEGFR1 soluble vascular endothelial growth factor receptor-1, TAN tumor-associated neutrophil, TEC tumor endothelial cell, TIMP tissue inhibitor of metalloproteinase, VEGF vascular endothelial growth factor, VEGFR2 vascular endothelial growth factor receptor-2, VSMC vascular smooth muscle cell

lar endothelial growth factor receptor-2 (VEGFR-2) in ECs that in turn binds to VEGF-A involved in blood vessel development [165]. The molecular mechanism consists in the proteolytic activation of PAR-1 by MMP-1. Then PAR-1 activates the nuclear factor κB (NF-κB) pathway (p65/RelA), and its p65 subunit binds to the kinase insert domain receptor (KDR)/VEFR2 promoter. Similarly, MMP-1 and MMP-2 together with EGFR/pan-HER ligand epiregulin (EREG) and cyclooxygenase 2 (COX2) are implicated in the formation of tortuous, dilated, and leaky new blood vessels [166]. These molecules are also involved in the increase of blood

vessel permeability and in tumor cell intravasation. MMP-7 also plays a role in angiogenesis. This MMP increases angiogenesis by the disruption of soluble VEGFR-1 blocking the sequestration of VEGF enhancing its bioavailability [167].

Regarding MMP-8, this MMP may participate indirectly in TEC activation and migration through the proteolytic cleavage of angiotensin I to angiotensin II [168]. Angiotensin II has the capacity of upregulating platelet endothelial cell adhesion molecule-1 (PECAM-1) expression that in turn favors β-catenin nuclear translocation and EC proliferation through the expression of CCND1, T-cell factor (TCF) 1B, TCF1E, and frizzled (FZD) genes. Moreover, MMP-8 knockdown in human umbilical vein endothelial cells (HuVECs) inhibits the expression of these genes and cell proliferation [168]. Furthermore, MMP-8 can induce EC migration through the expression of EMT genes regulated by β-catenin (see EMT section). Likewise, MMP-9 makes an important contribution to angiogenesis. MMP-9 from tumoral cells, CAFs, and TANs is able to disrupt the ECM releasing VEGF. In this context, pro-MMP-9 from TANs is not in complex with TIMP-1 allowing a rapid activation by MMP-3 with the subsequent activation of the pro-angiogenic factor bFGF-2 that is also embedded in the ECM. This action of TANs' MMP-9 occurs faster than the effect caused by MMP-9 from other sources [99]. Likewise, MMP-9 favors EC migration and allows sprouting angiogenesis through the degradation of multimerin-2 (MMRN2) from the EC surface [169]. MMRN2 is involved in the disruption of the VEGFA/VEGFR2 signaling axis suppressing EC movement and angiogenesis progression [170]. Additionally, experiments done in MMP-$9^{-/-}$mice with neuroblastoma showed that new blood vessels were smaller in size and lower in number with a decrease in pericyte recruitment in comparison with MMP-$9^{+/+}$ [171]. These authors also observed in neuroblastoma samples that MMP-9 was located mainly around blood vessels particularly in ECs suggesting its participation in vessel maturation. Similarly, when TECs are activated, they degrade BM components trough the secretion of MMP-2 and MMP-9 to migrate to where angiogenesis is taking place driven by tip cells [164]. Additionally, TECs have high amounts of uPAR involved in the activation of plasminogen to plasmin. Plasmin is one of the physiological activators of pro-MMPs [26].

Likewise, MMP-14 is increased in TEC membrane where it favors pro-MMP-2 activation and TEC migration. Moreover, MMP-14 also participates in vascular tunnel formation creating spaces in the ECM called vascular guidance tunnels by the degradation of ECM components [172]. MMP-14 also favors the formation of TEC aggregates that participate in lumen formation. Functions of MMP-14 depend on the endothelial signaling complex formed by Jam-B, Jam-C, Cdc42-GTP, α2β1 integrin, and MMP-14. This complex regulates tubulogenesis [172]. Once the vascular guidance tunnel network is created, TECs regulate tube assembly and vascular remodeling. TECs also produce MMP-1 and MMP-10 that may induce vascular regression and/or the collapse of vascular guidance tunnels and tubes [172]. To avoid this effect and protect and stabilize the new vascular tubes, pericytes secrete TIMP-3 while TECs release TIMP-2 that also has the capacity to block MMP-2 and MMP-14 suppressing type IV collagen degradation [173]. Moreover, pericytes with ECs are responsible for new vessel BM assembly by the secretion of ECM molecules such as type IV collagen, nidogen-1, nidogen-2, laminin, perlecan, and fibronectin [174]. Additionally, MMP-14 facilitates the dedifferentiation of vascular smooth muscle cells (VSMCs) that contribute to vessel maturation [175]. In this context, MMP-14 disrupts low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) decreasing platelet-derived growth factor receptor β (PDGFRβ) polyubiquitin-directed degradation. Stimulation of PDGFRβ by platelet-derived growth factor-BB (PDGF-BB) downregulates the expression of contractile proteins such as calponin and SMA with the increase of vimentin transforming VSMCs from a contractile to a migrant phenotype. Furthermore, MMP-14 also increases PDGFRβ internalization in caveolae [175].

Notwithstanding, not all MMPs have an angiogenic function. Such is the case of MMP-19 that is expressed in the early stages of breast, skin, and colon cancer but is downregulated during the disease progression, probably because of its anti-angiogenic function. In this context, MMP-19 expression was decreased in primary and metastatic nasopharyngeal carcinoma tumors as well as in cell lines due to promoter hypermethylation and to allelic detections [176]. Likewise, experiments done in HuVECs and HMEC-1 cells (dermal endothelial cells) cultured with CM from nasopharyngeal carcinoma cells transfected with MMP-19 mutated in its catalytic region showed an increase in their vascular tube formation capacities while the wild-type MMP-19 has the opposite effects including VEGF synthesis inhibition [176]. These experiments demonstrated that the MMP-19's anti-angiogenic properties depend on its catalytic site. Moreover, MMP-19 also interferes with VEGF displacement from the ECM reducing its bioavailability. In contrast, other authors have identified that MMP-3, MMP-7, MMP-9, and MMP-19, and in a lower degree MMP-1 and MMP-16, cleave VEGFA releasing a 16-kDa fragment [177]. Moreover, this proteolytic event separates the VEGFA receptor binding domain from the ECM-attaching motif, and the soluble fragments generated in this way are able to phosphorylate the VEGFR promoting angiogenesis [177]. Interestingly, not all VEGF isoforms are susceptible to MMPs' processing and therefore have a different angiogenic behavior. Tumors that are VEGF MMP-resistant have vascular sprouting and branching with the organization of cords, while VEGF fragments correlate with endothelial migration and cell growing as sheets [177]. These findings do not discard the possibility that MMPs are able to release VEGF from the ECM during the angiogenesis process.

On the other hand, as was mentioned above, angiogenesis must be controlled to prevent vascular regression and protect new vessels' integrity. Besides TIMPs' participation, MMPs' activation is regulated by the presence of plasminogen activator inhibitor (PAI)-1 and PAI-2 that interfere with plasmin production [178]. Likewise, platelet factor-4 (PF4) suppresses the effect of thrombin in the upregulation of MMP-1 and MMP-3 expression blocking EC migration. Angiostatin is also involved in angiogenesis control. This molecule is the product of plasminogen cleavage and, besides other anti-angiogenic proprieties, has the ability to inhibit EC mobilization through the downregulation of MMP-2 and MMP-14 in hypoxic conditions [179].

Additionally, disruption of the BM and ECM components, such as laminin, elastin, type IV collagen, proteoglycans, and fibronectin, by the activity of different proteases including MMPs, releases protein motifs known as matrikines and unmasks cryptic sites in the ECM called matricryptins [180, 181]. Matrikines and matricryptins are involved in the regulation of several processes during cancer progression. Regarding angiogenesis, matricryptins and matrikines can provoke TEC apoptosis and suppress TEC proliferation, mobilization, and tube construction [182]. Interestingly, TECs synthesize and secrete MMPs that degrade collagens type IV, XV, XVIII, and XIX, perlecan, and laminin, whose proteolytic products have an anti-angiogenic effect [183]. This TEC behavior probably is part of the molecular mechanism involved in angiogenesis regulation. For example, endostatin, a matrikine, forms a complex with MMP-2 catalytic motif with the subsequent loss of its enzymatic activity and a decrease in tumor and endothelial invasiveness [184]. For a major review regarding matricryptins and matrikines with anti-angiogenic effects, see Gonzalez-Avila G et al. [6]. Among the MMPs that participate in proteolysis of BM and ECM are MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, and MMP-20 [125, 181, 185].

Reasonably, communication among cells during angiogenesis is regulated by molecules contained in the EVs. For instance, tumor-derived EVs may contain pro-angiogenic factors such as VEGF, sphingomyelin, IL-6, IL-8, FGF, and miRNAs [186]. Remarkably, MMP-2, MMP-9, and MMP-14 expressed by ECs may be stored in cytoplasmic secretory granules and are released in EVs [187]. Moreover, VEGF and FGF increase

the number of EVs containing MMPs and stimulate a fast EV shedding. These growth factors also participate in MMP enzymatic activation. EV-associated MMPs have an autocrine effect on TECs increasing their migration and tubular structure formation abilities [187]. EC-derived EVs also contain β1 integrin for their interaction with the surrounding ECM. Likewise, tumor-derived EVs that contain the MMP inducer CD147 promote HuVECs' MMP-1, MMP-2, and MMP-14 expression and increase their invasiveness and cord formation capacities [188]. Furthermore, EVs derived from ASCs stimulated with PDGF contain MMP-2 and MMP-9 that favor EC migration [186].

5.8.2 Lymphangiogenesis

Lymph vessels (LVs) are another route that neoplastic cells use to escape from the primary TME. As in the case of angiogenesis, lymphangiogenesis consists on the creation of new LVs from preexisting LVs or from veins [189]. In contrast to new blood vessels, LVs are irregular and leaky structures in which gaps between lymphatic endothelial cells (LECs) exist; they are devoid of pericyte and VSMC layers and have a poor deposit of BM components [189, 190].

Two mechanisms that may stimulate lymphangiogenesis have been identified. The first consists in cancer cell recruitment of endothelial progenitor cells from bone marrow that express VEGFR-3 with their later integration to the LV extensions. The second process involves the interaction of pro-lymphangiogenic factors with LECs from preexisting LVs [191]. Interestingly, there is evidence that, under the influence of tumor cells, macrophages may transdifferentiate into LECs, initially generating cell aggregates followed by vesicle formation and their integration into sprouting LVs [192]. However, macrophages exposed to pro-inflammatory molecules such as TNFα enhance the expression and release of VEGF-C favoring the sprouting of preexisting LVs [192]. Neoplastic cells induce lymphangiogenesis by the release of HGF, VEGF-A, VEGF-C, and VEGF-D [193]. Likewise, other factors may also participate such as FGF-2, insulin-like growth factor-1 (IGF-1), IGF-2, endothelin-1 (Et-1), and PDGF-BB secreted by other cells, although the most relevant pro-lymphangiogenic factor is VEGF-C/VEGF-D/VEGFR-3 [193]. Active VEGFR-3 promotes LEC proliferation and migration and prevents their apoptosis through the activation of Akt, ERK, and p42/p44 MAPK pathways [194]. Additionally, VEGFR-3 interacts directly with PI3K inducing its phosphorylation that stimulates LEC migration and tube formation [195]. VEGF-C and VEGF-D also attach to neuropilin-2 (NRP-2) that is co-internalized with VEGFR-3. Both molecules form a complex that drives initial lymphatic sprouting [189, 195]. Similarly, the binding of ET-1 to its receptor E_B favors LEC proliferation and migration through the activation of p42/44 MAPK and Akt signaling pathways and by MMP-2 and MMP-9 expression that play a role in tube formation [196]. Notably, during LV formation MMP-2 can disrupt type I collagen matrix allowing LEC mobilization through this ECM component [197]. Moreover, lower MMP-2 expression alters LV formation and branching in zebrafish and mice models. Additionally, the use of the MMPs' competitive inhibitor SB-3CT suppresses MMP-2 and MMP-9 activity and downregulates VEGF-C and VEGFR-3 expression inducing a decrease in lymphangiogenesis in a corneal model [198]. Likewise, MMP-13 increases VEGF-C expression through the activation of PI3K/Akt signaling pathway favoring lymphangiogenesis and tumor metastasis [199].

MMP-14 also participates in lymphangiogenesis although with controversial roles. On the one hand, MMP-14 favors lymphangiogenesis by inducing LEC migration and LV sprouting, besides its role in pro-MMP-2 activation [200]. On the other hand, MMP-14 cuts off the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) from LEC surface interfering with lymphangiogenesis signaling since LYVE-1 binding to hyaluronic acid (HA) drives LEC growth [201]. Moreover, MMP-14 also suppresses macrophage VEGF-C expression through its binding to PI3K promoter with the subsequent

p110δ upregulation [201]. PI3Kδ in turn blocks NF-κB nuclear translocation and VEGF-C synthesis. In this regard, MMP-16 may serve as a pro-lymphangiogenic factor since this MMP sheds MMP-14 from cell surface promoting LEC invasion [202]. Moreover, MMP-16 downregulation switches cancer progression from a lymphatic to a blood invasion process in tumors produced by the implantation of melanoma cells in mice.

5.9 Comments and Future Trends

TME metabolic conditions as well as several non-neoplastic cells contribute to provide tumor cells with characteristics that allow them to proceed with the following steps of the metastatic cascade. Cancer cells acquire the ability to invade the BM and ECM components to reach new lymphatic and blood vessels. In both invasion and intravasation, MMPs play a paramount role. Likewise, tumor invasiveness, neoplastic cell morphology (spike, amoeboid, or mesenchymal forms), migration strategies as single or cell groups, and MMPs' expression are dependent on ECM stiffness and composition [203–205]. For instance, cancer cell interaction with type IV collagen promotes MMP-2 and MMP-9 release while MMP-1 and MMP-13 are secreted when cells bind to type I collagen [206, 207]. Meanwhile, during invasion MMPs are located in membrane extensions such as blebs, filopodia, and invadopodia [208–210]. In this context, presence of β1 integrin mediates localization of MMP-2, MMP-9, and MMP-14 in invadopodia to disrupt BM structures. Moreover, cells such as CAFs construct paths where tumor cells transit during invasion (see above). Once cancer cells arrive to blood or lymphatic vessels, they intravasate and travel to different tissues to establish a new metastatic colony with an active participation of MMPs in all this journey [6]. Therefore, MMPs' expression has been associated with tumor cell aggressiveness and metastatic potential, and consequently these enzymes are considered as bad prognosis biomarkers. However, some MMPs have protective effects particularly at the disease's early stages. Such is the case of MMP-3, MMP-8, MMP-12, MMP-13, MMP-19, and MMP-26 [6]. Interestingly, MMP-11 has pro-cancer functions during the early stages of illness due to its anti-apoptotic properties and its effects on adipocytes (see above), but it also has metastatic protective capacities during late stages [211, 212]. Likewise, there are differences in MMPs' expression and functions among cancer types. Therefore, it is important to establish a specific MMPs' profile for each case taking into account the stage of the disease.

On the other hand, since MMPs play a role in all the steps of cancer dissemination, they have been considered as therapeutic targets. In this context, several strategies have been developed to inhibit their enzymatic activity [213, 214]. However, the expected success has not been achieved due to side effects produced by the MMPs' inhibitors and to the lack of improvement in patients' survival rates. Moreover, matrix metalloproteinase inhibitors are not designed to block specific MMPs; therefore, MMPs with protective effects are also targets for these drugs with the subsequent worsening of the disease. Nevertheless, the expectation of inhibiting MMPs' functions led to the creation of alternative methods to drive a specific drug to a specific tissue, such as the use of nanotechnology [215]. In this context, several delivery systems have been developed to control different TME elements and processes including TAMs, CAFs, and angiogenesis [216]. Furthermore, nanotechnology has been used in the theranostic approach that allows specific diagnosis, treatment, and monitoring of therapy response [217]. For example, the inclusion of a cytotoxic drug such as docetaxel or paclitaxel with a chemical sequence that is cleaved by MMP-2 or MMP-9 in a nanosystem targeted to a specific tumor that overexpresses these MMPs has improved patients' illness outcomes with fewer side effects [218, 219].

In summary, MMPs have a relevant role in TME with their participation in processes that allow neoplastic cells to acquire characteristics that secure their survival in adverse microenvironmental conditions. Moreover, MMPs provide

the escape mechanisms for cancer cells to detach from the primary tumor and invade the surrounding tissue searching for the way that conducts them to a new organ. Furthermore, MMPs also participate in the construction of these routes. Therefore, an early disease diagnosis with the knowledge of which MMPs are implicated in the TME may aid in controlling cancer progression in the very early stages of the disease.

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6 Flipping the Molecular Switch: Influence of Perlecan and Its Modifiers in the Tumor Microenvironment

Lissette A. Cruz, Tristen V. Tellman, and Mary C. Farach-Carson

Abstract

The tumor microenvironment (TME) is rich in matrix components, growth factors, cytokines, and enzymatic modifiers that respond to changing conditions, to alter the fundamental properties of the tumor bed. Perlecan/HSPG2, a large, multi-domain heparan sulfate proteoglycan, is concentrated in the reactive stroma that surrounds tumors. Depending on its state in the TME, perlecan can either prevent or promote the progression of cancers to metastatic disease. Breast, prostate, lung, and renal cancers all preferentially metastasize to bone, a dense, perlecan-rich environment that is initially a "hostile" niche for cancer cells. Driven by inflammation, production of perlecan and its enzyme modifiers, which include matrix metalloproteinases (MMPs), sulfatases (SULFs), and heparanase (HPSE), increases in the reactive stroma surrounding growing and invading tumors. MMPs act upon the perlecan core protein, releasing bioactive fragments of the protein, primarily from C-terminal domains IV and V. These fragments influence cell adhesion, invasion, and angiogenesis. Sulfatases and heparanases act directly upon the heparan sulfate chains, releasing growth factors from reservoirs to reach receptors on the cancer cell surface. We propose that perlecan modifiers, by promoting the degradation of the perlecan-rich stroma, "flip the molecular switch" and convert the "hostile" stroma into a welcoming one that supports cancer dissemination and metastasis. Targeted therapies that prevent this molecular conversion of the TME should be considered as potential new therapeutics to limit metastasis.

Keywords

Perlecan/HSPG2 · Heparan sulfate proteoglycan · Matrix metalloproteinase · Sulfatase · Heparanase · Bone · Inflammation · Metastasis · Glycocalyx · Breast · Prostate · Lung · Renal · Reactive stroma

Lissette A. Cruz and Tristen V. Tellman contributed equally.

L. A. Cruz · T. V. Tellman · M. C. Farach-Carson (✉)
Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston, Houston, TX, USA
e-mail: Lissette.Cruz@uth.tmc.edu; Tristen.Tellman@uth.tmc.edu; Mary.C.FarachCarson@uth.tmc.edu

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6.1 Introduction to the Tumor Microenvironment (TME)

6.1.1 Components of the TME

As the cancer field shifts toward a macroscopic view of the tumor microenvironment (TME), the need to understand the complexities of tumor-stromal interactions moves to the forefront [1]. Cellular and molecular interactions at the cancer cell surface relay a constant stream of signals that influence cancer growth and metastasis. The TME consists of the non-cancerous cells present in and around the tumor including fibroblasts, immune cells, and endothelial cells in conjunction with extracellular matrices (ECM) that can support growth, survival, and metastasis of cancer cells. These matrices, factors that bind to them, and their enzymatic modifiers can be produced by any of the cells present in the TME, working dynamically either to prevent or promote cancer cell dissemination (Fig. 6.1).

6.1.2 Role of TME in Cancer Progression

While the exact nature of the cancer-stromal interaction is still being defined, work in many labs has begun to illustrate the enormous impact the TME can have on cancer progression. For example, Yu-Lee et al. conducted a systematic study in mice, inoculating similar numbers of prostate cancer cells into two locations: subcutaneously in the back and intrafemorally. A comparison of the outcomes between these two groups revealed significantly less growth for tumors growing in the bone versus their skin counterparts. Specifically, cells in the bone, a perlecan-rich environment, became dormant, whereas those in the skin formed tumors within 3–5 weeks [2]. This finding can only be explained in the context of the cancer-stromal interface, highlighting the importance of localized TME, especially in bone, on key aspects of cancer cell behavior.

Breast, prostate, lung, and renal cancers represent about 75% of all cancers that preferentially metastasize to bone [3, 4]. The bone represents a unique TME that, once colonized, is associated

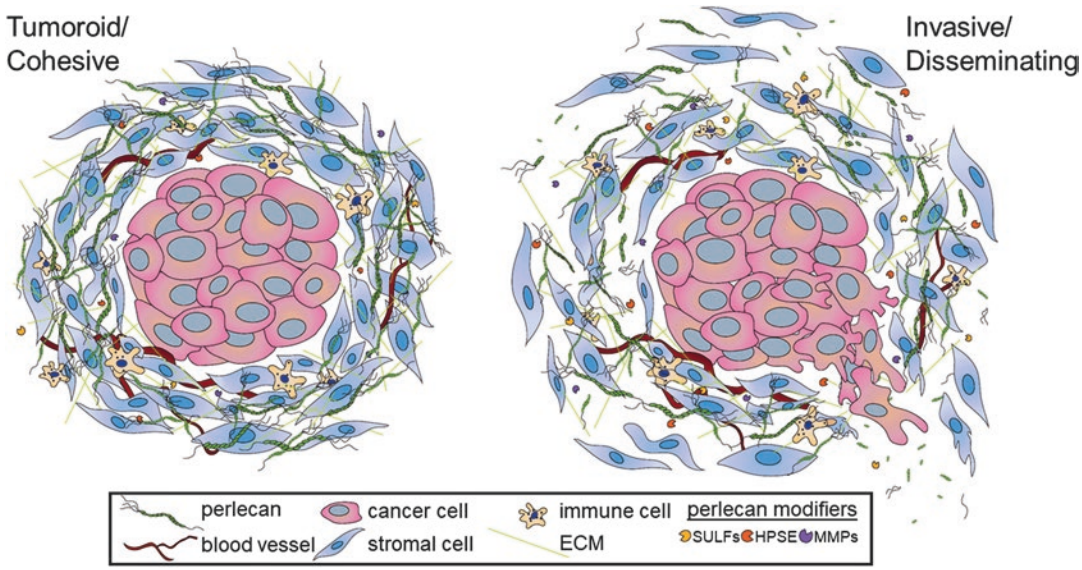

Fig. 6.1 Dynamic interactions among various cell types present in the TME. Reactive stroma (left panel) includes perlecan that attempts to "wall off" the tumor, limiting invasion and preventing dissemination. Disseminating tumors remodel the ECM, including cleavage of perlecan, allowing for tumor dyscohesion and invasion

with progressive metastasis and often lethal disease [5]. Given that the TME in bone is initially "hostile" to invading cancer cells, it is interesting to consider how an initially dormancy-inducing TME becomes one that fosters cancer cell growth and metastasis [6]. Cancers metastasizing to bone can be divided into two subtypes: osteolytic and osteoblastic/sclerotic. Osteolytic bone metastases are responsible for the destruction of bone, while those of the osteoblastic subtype are considered bone-forming. Prostate cancer bone metastases are most often osteoblastic, though some more neuroendocrine tumors can produce a mixed population of osteoblastic and osteolytic lesions [7]. Bone metastases from primary renal, lung, and breast tumors have a tendency to be more osteolytic, where osteoclasts are controlled by the invading cancer cells [8, 9]. In each case, the TME plays a vital role in determination of the type of lesion that will form and how the cancer will progress.

6.1.3 Extracellular Matrix in the TME

Researchers have begun to appreciate the impact that various ECM constituents in the TME can have on normal and disease biology [1]. In traditional wound healing responses, ECM remodeling and growth factor actions bring damaged tissue back to homeostasis. These normal processes are pathologically co-opted by cancer cells in the TME, leading to its description as the "wound that never heals" [10]. As recently defined in the Matrisome Project, the ECM is composed of 274 core proteins with 753 associated factors, proteins, and regulators, each functioning to maintain tissue integrity and to provide a reservoir of readily available factors to promote wound healing and regeneration [11]. Each tissue expresses a unique subset of these components that comprise the TME. In cancer, these ECM components in the TME can become major drivers or inhibitors of metastasis and disease progression. Proteoglycans are core components of the Matrisome that are hallmarked by their structural and functional diversity, play major roles in cancer cell fate.

Proteoglycans are defined as proteins containing one or more covalently attached glycosaminoglycan (GAG) chains. GAG chains are categorized into four major classes, heparan sulfate, chondroitin sulfate, keratan sulfate, and hyaluronate, the last of which is synthesized as a free glycan [12]. The composition of these GAG chains on proteoglycans varies greatly within different tissues, with some predominated by heparan sulfate and others by chondroitin or keratan sulfate. Among these GAGs, heparan sulfate plays an essential role in the binding of heparin-binding growth factors (HBGFs) and is composed of unbranched negatively charged disaccharide units with spatially organized sulfate groups to endow binding specificity for individual HBGFs [13]. Release of these HBGFs relies upon three key groups of extracellular enzymes that can modify heparan sulfate polymers and alter growth factor binding and local bioavailability: matrix metalloproteinases (MMPs), sulfatases (SULF1 and SULF2), and heparanase (HPSE) [14].

6.2 The TME, Glycocalyx, and Pericellular Matrix

6.2.1 TME and Cancer Cell Behavior

Tumors are not just masses of clustered malignant cells, but rather they can be considered as "disorganoids" that are composed of various cell types, including fibroblasts, stromal cells, immune cells, and cells from the vascular network that are encased by a dense ECM in the pericellular space. Cancer cells not only depend on driver oncogenes to survive, grow, and metastasize, but they also rely on pro-survival signals produced in the associated stroma [15]. Despite their growth persistence, highly aneuploid, genetically unstable cancer cells are often quite fragile and die rapidly when separated from the TME to which they have become accustomed.

6.2.2 Glycocalyx

The "glycocalyx," another component of the TME, is a layer of glycans present on the surface of cancer cells as well as various normal cell types and tissue structures [12]. The glycocalyx serves a variety of functions that both protect cells and ensure their survival. Heparan sulfate proteoglycans (HSPGs), such as syndecan and glypican, are present at the cell surface where they often function as co-receptors for growth factor signaling complexes. For example, binding of fibroblast growth factor-2 (FGF-2/basic FGF) to its receptor is stabilized by heparan sulfate found on the GAG chains of the co-receptor, typically syndecan [16]. Acting at or near the cell surface, extracellular modifiers of heparan sulfate such as the SULFs and HPSE can play vital roles in modulation of growth factor signaling, cell survival, invasion, and metastasis.

6.2.3 Pericellular Matrix

As cancer progresses, normal tissue boundaries are disrupted and local ECM turnover prevails. Among these ECM components, heparan sulfate proteoglycan 2 (HSPG2)/perlecan, a major component of the basement membrane, is critically involved in patrolling tissue boundaries [17]. Perlecan can be produced by some cancer cells, but the majority of perlecan in the TME is made by cells in the reactive stroma where it co-localizes with smooth muscle actin, tenascin, and thrombospondins [17, 18]. Unlike the HSPGs syndecan and glypican that reside in the glycocalyx of cancer cells, perlecan is fully secreted and resides in the pericellular space [12]. Perlecan is present at high levels in the reactive stroma surrounding breast, lung, renal, and prostate cancer lesions (Fig. 6.2). Perlecan modification by SULF1, SULF2, or HPSE in the TME affects cancer cell proliferation, survival, invasion, and metastasis [12, 14]. Upon injury or invasion that penetrates the basement membrane, cancer cells come in contact with the cells in the stromal compartment of the TME. In stroma, bound HBGFs can be released enzymatically from perlecan bound and sequestered in the stroma. This occurs as a direct consequence of the activation of various matrix remodeling enzymes in the TME that include both proteases and glycosaminoglycanases. These degradative processes continue during metastasis, such as to bone, where they foster the development of secondary and tertiary metastases. In this chapter, we will focus specifically on three of these extracellular enzyme modifiers of perlecan: MMPs, SULFs, and HPSE. Each of these enzymes plays a role in the TME during initial cancer invasion and metastasis and then later in the metastatic niche of bone or other common sites of secondary cancer growth.

6.3 Perlecan/HSPG2 in the Tumor Microenvironment

6.3.1 Perlecan Function in the TME

In the presence of transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α) is a major cytokine regulator of perlecan mRNA expression in cancer cells, normal stromal cells, and a subpopulation of bone marrow stromal cells [19]. In the context of breast cancer, the perlecan promoter can be positively regulated by TGF-β and negatively regulated by interferon-γ (INF-γ) [20]. TGF-β and TNF-α recruit and activate immune, endothelial, and stromal cells at the primary tumor or metastatic sites; this process, in turn, further triggers production of inflammatory cytokines and ECM, creating a positive feedforward loop. In normal tissues, perlecan possesses antitumoral activity by stabilizing tissue borders, decreasing cell motility, and favoring cell survival. Epithelial cells, epidermal cells, endothelial cells, smooth muscle cells, fibroblasts, osteocytes, and chondrocytes all can synthesize perlecan [17]. These various perlecan cellular sources contribute to the distribution of perlecan in the basement membrane, in the stromal matrix, and at other tissue borders including in bone [17]. It is well known that an intact epithelial basement membrane exists in benign tumors, whereas

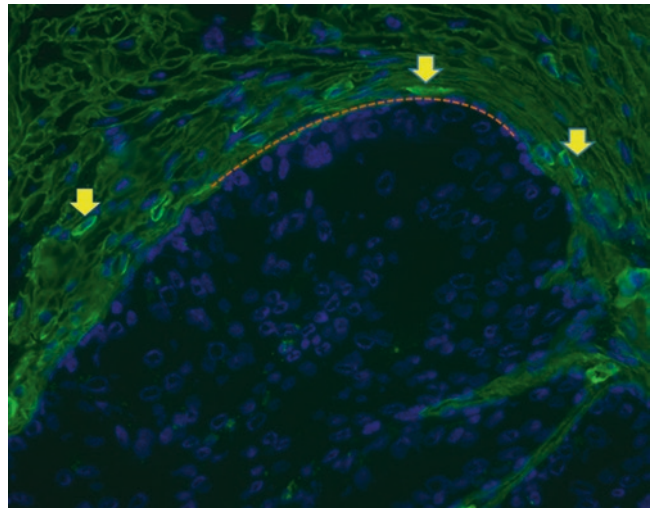

Fig. 6.2 Immunofluorescence staining of perlecan-rich stroma in the TME surrounding a primary prostatic lesion. Dotted line indicates start of the non-permissive perlecan barrier adjacent to the basement membrane surrounding the lesion. Perlecan (green) and nuclei (blue). Note the intense staining of perlecan surrounding the blood vessels near the tumor (arrowheads)

invasive tumors lack an intact basement membrane allowing cells to move into stroma [21]. Perlecan expression is highly regulated in the TME surrounding invasive and metastatic carcinomas, specifically in the desmoplastic stroma and at sites of bone metastasis [19, 22–26]. Also, perlecan expression is induced in various tumors, particularly those undergoing epithelial-to-mesenchymal transition (EMT) [27]. Studies have indicated that metastatic tumors might be detected by the host defense system, and these tumors are encapsulated with dense perlecan-rich matrix to prevent further dissemination of these tumor cells [19, 28]. Evidence suggests it is likely that tumor cells and cells in the stroma defeat this barrier function over time by expressing enzymes that participate in basement membrane degradation, such as MMPs, SULFs, and HPSE.

Until recently, the identity of a direct binding partner of perlecan at the cancer cell surface remained elusive. A recent study from our lab found that semaphorin 3A (SEMA3A) and the most C-terminal portion of the fourth domain of perlecan, domain IV-3, interact with one another to induce prostate cancer cell-cell cohesion and dissolution of focal adhesions [29]. Work done by Herman et al. showed the strong influence of SEMA3A in the TME surrounding prostate cancer cells, where it inhibits migration and invasion [30]. The recently described interaction of SEMA3A with perlecan may explain the similar phenotypes observed when both molecules are dynamically altered in the TME [29].

Perlecan in the TME not only acts as a physical barrier to restrict cell movement, but its heparan sulfate chains also sequester bioactive proteins such as HBGFs, chemokines, cytokines, and some enzymes, adding to the complexity of perlecan's role in tissue remodeling and understanding of its role in tumor progression [27]. A wide variety of HBGFs form complexes with perlecan, such as members of the fibroblast growth factor family, vascular endothelial growth factor, heparin-binding EGF, and many cytokines (e.g., interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and INF-γ [31]. FGF2 is sequestered in complex with perlecan in the basement membrane and stroma of various tissues and by other HSPGs in the glycocalyx [32, 33]. Perlecan's heparan sulfate chains typically sequester the FGF ligand, although interactions of other FGFs with the core protein have been reported [34]. The release of the FGF ligand from sequestration

sites in stroma allows for diffusion to receptor binding sites in the glycocalyx to activate complex signaling cascades that control cell proliferation, motility, and adhesion [12, 35].

6.3.2 Perlecan and Angiogenesis

Neoangiogenesis, the development of new blood vessels from pre-existing vasculature, is required in early tumorigenesis to supply nutrients and oxygen to cancer cells [12, 36, 37]. Angiogenesis in the TME is a complex process, which involves the organized actions of pericytes, endothelial cells, and smooth muscle cells [20]. A group of major players needed for malignant angiogenesis is the family of MMPs (Table 6.1) [36, 38]. Proteolytic release of the C-terminal region of perlecan produces fragments with dramatic effects on angiogenesis [39]. These fragments, known variously as endorepellin, domain V, and C-terminal laminin-like globular domain (LG3), remain a very active area of study that may lead to production of novel classes of therapeutics for a variety of angiogenic-related disorders [40, 41].

6.4 Immune Cells in the TME

6.4.1 Immune Cells and Cytokines

Immune infiltration and resulting inflammation are hallmark features of a reactive stromal response. Chronic inflammation is a major driver of ECM deposition and catabolic enzyme upregulation, with a net overall effect of increased tissue turnover. This turnover digests the matrix-bound core protein and releases diffusible perlecan fragments that can have activities distinct from the intact proteoglycan. Peptide mapping showed the majority of these fragments are derived from the C-terminus and can be detected in the blood of patients with metastases [23]. Various studies found macrophages to represent a large portion of the diverse immune infiltration population in the TME. Tumor-associated macrophages (TAMs) in advanced disease phenotypically resemble M2 macrophages, often

Table 6.1 Major enzyme modifiers of perlecan/HSPG2 in the TME

Gene/protein	Activity	Major sources in TME	References
MMP-1/interstitial collagenase	Zinc-dependent endopeptidase Produces multiple small peptides when used with heparitinase/chondroitinase Degrades a variety of matrix components	S	[38, 50, 51]
MMP-3/stromelysin	Zinc-dependent endopeptidase Produces multiple small peptides when used with heparitinase/chondroitinase Degrades a variety of matrix components including perlecan	S, E	[38, 52]
MMP-7/matrilysin/epithelial MMP	Zinc-dependent endopeptidase Produces multiple small peptides even in the presence of HS/CS chains Degrades a variety of matrix components including perlecan	CC	[53–56]
SULF1	Possesses endoglucosamine-6-sulfatase activity Removes 6-O-sulfate from HS chains on HSPGs to alter interactions with HBGFs	F, S	[57, 58]
SULF2	Possesses endoglucosamine-6-sulfatase activity Removes 6-O-sulfate from HS chains on HSPGs to alter interactions with HBGFs	F, S	[57, 58]
HPSE	Endo-β-D-glucuronidase that cleaves HS chains on HSPGs to produce small fragments that may bear bound HBGFs	CC	[59]

S Stromal cells, *E* endothelial cells, *CC* cancer cells, *F* fibroblasts, *HBGFs* heparin-binding growth factors, *HS* heparan sulfate, *HSPGs* heparan sulfate proteoglycans

stimulating and promoting neovascularization and the induction of vascular network formation [42]. Interestingly, high levels of TAM infiltration are associated with poor patient survival and dim prognosis in patients with lung, breast, renal, or prostate cancers. The presence of these TAMs in the TME can exacerbate chronic inflammation and stimulate ECM remodeling, paralleling events that would occur in wound healing [43]. While TAMs are the most abundant immune cell type in the TME, several reports in prostate cancer show an increased presence of other immune cells including myeloid-derived suppressor cells and natural killer cells, all potentially conferring the innate TME immune response [44]. Ongoing work aims to determine how the presence of these various classes of immune cells in the TME contribute to the transition from a "hostile" TME to one that participates and accelerates metastasis and lethal progression.

6.4.2 Inflammation in the TME

TNF-α, a protein often present during inflammation, is present at high levels in the tumor microenvironment of prostate, breast, lung, and renal cancers [45, 46]. TNF-α can be produced by many cell types in the TME, but it is most commonly known as a factor released by TAMs. TNF-α released by immune cells in the prostate cancer microenvironment increases the expression and secretion of perlecan by both prostate cancer cells and bone stromal cells via TNF-α-induced nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) translocation to the nucleus. Once inside the nucleus, NFκB undergoes a unique binding step, where binding to the *HSPG2* promoter region increases perlecan transcript levels [19]. In breast cancer, the tumor microenvironment demonstrates a similar phenomenon, where TNF-α released from reactive stroma in breast cancer signals for increased expression of ECM proteins and ECM remodeling enzymes [46]. Similarly, TNF-α, interleukin-1β (IL-1β) , and interleukin-6 (IL-6) all contribute to cancer metastasis through induced secretion of HPSE from endothelial cells in the TME. The presence of HPSE further favors EMT and enhances pro-metastatic signaling [47, 48].Along with HPSE, other remodeling enzymes (e.g., MMPs, SULFs) can be activated by resulting inflammation from infiltrating immune cells. Hagemann et al. published a co-culture study with TAMs and invasive breast cancer cell lines, observing an increase in MMP-2, MMP-3, MMP-7, and MMP-9 in a TNF-α-dependent manner [49]. In the TME, this TAM-mediated inflammation plays various roles, upregulating not just the production of perlecan but also perlecan-modifying enzymes, ultimately helping to flip the "molecular switch."

6.5 Perlecan Modifiers in the TME

The reactive TME is rich in perlecan and its enzyme modifiers whose expression is regulated by environmental factors such as inflammation, factors produced by the disseminated cancer cells themselves, tissue turnover, and the unique character of the tumor site. Table 6.1 provides a summary of some of the more common enzyme modifiers found in the TME that influence the molecular state of perlecan and that together comprise the molecular switch responsible for converting a TME from "hostile" to one that actively participates in tumor growth and metastasis. Thus, these modifiers can be considered to be the factors that "flip the switch" from conditions that limit progression to those that favor further metastasis and onset of lethal disease.

6.5.1 MMPs

It is well accepted that MMPs are upregulated in many cancers, especially in the presence of chronic inflammation. These MMPs have been studied for decades for their capacity to degrade and remodel surrounding matrix in the TME, fostering invasive and metastatic disease. MMP-1, MMP-3, and MMP-7 can digest perlecan in the TME, but for MMP-1 and MMP-3, the efficient removal of heparan sulfate or chondroitin sulfate

chains first must occur [38, 53]. MMP-7 demonstrates a unique ability to degrade perlecan without prior removal of the GAG chains, a feature that contributes to its overall impact in the degradation of basement membranes and destruction of reactive stroma [53]. In 2009, work done by the Parks group demonstrated an interesting interplay between heparan sulfate chains and the proteolytic activity of MMP-7, showing that sulfated GAGs can drive activity and specificity of the MMP [60]. While each of these MMPs plays vital roles in contributing to the whole cancer TME landscape, their localization often differs. MMP-7 more frequently localizes to the luminal cancer cell compartment while MMP-1, MMP-2, and MMP-9 tend to localize specifically to the stromal cells [61]. MMP-7 status in renal cell carcinoma, and other cancers, is a major indicator of disease progression and prognosis [23, 62]. In renal cell carcinomas, MMP-2 and MMP-9 showed increased expression in relation to their normal counterparts [63]. Interestingly, in a mouse model of prostate cancer, mice without MMP-2 showed increased survival outcome measures, while those with deficient MMP-7 demonstrated no significant changes in survival outcomes but showed a reduction in both endothelial area coverage and vessel size. In this same study, mice with deficient MMP-9 showed similar numbers of vessels within the tumor as compared to the control but demonstrated a decrease in vessel size, with a more elongated and regular vessel shape, illustrating the impact that various MMPs can have on tumor angiogenesis and survival [61]. Considered together, in a survey of the breast, lung, prostate, and renal literature, it is generally true that elevated levels of MMPs correlate with poor prognosis for patients. Knowing this, it seems that targeting MMPs would be an effective method to control the progression of cancers to metastatic and, ultimately, lethal disease. Marimastat, a competitive MMP inhibitor, underwent clinical trial in both breast and small-cell lung cancer, failing in both settings. When compared to the placebo group, patients receiving Marimastat treatment showed no significant benefit in progression-free survival, and in some cases, treatment resulted in inferior overall patient health due to musculoskeletal toxicity [64, 65]. While we choose to highlight Marimastat in this chapter, other clinical trials with MMP inhibitors have yielded similar results, illustrating their ineffectiveness as a singular therapy [66]. These inhibitors have not yet been discarded as an option for treatment however, as combinatorial therapies with other compounds, such as Carboplatin, have yielded promising preliminary results [67].

6.5.2 SULFs

Studies of the molecular composition of the TME have shown that extracellular sulfatases frequently reside in the stroma surrounding growing tumors where they can act directly on perlecan deposited there. Sulfatase (SULF) expression patterns in the TME are complex, with different cancers demonstrating unique SULF signatures. It is interesting to think about the impact of SULF localization in explaining these unique signatures in the TME. SULFs localized at the cancer cell surface would have a large negative impact on the ability of cell surface heparan sulfate proteoglycans in the glycocalyx such as syndecan and glypican to act in their co-receptor roles. SULFs acting at this location release growth factors away from the cell surface, suppressing growth and creating growth factor reservoirs in the surrounding stroma. In contrast, those SULFs localized in the stromal compartment release available HBGFs from the stroma to diffuse and bind their specific receptors at the cell surface. These opposing actions ultimately promote localized growth of the cancer cells even in the presence of SULFs in the glycolax. Studies performed using various cancer cell types demonstrate the dynamic influence of SULFs on the invasion and growth potential for cancers. For example, in reports using breast cancer cells, SULF1 expression is reduced while SULF2 is upregulated in the localized tumor [68, 69]. Lung cancer SULF expression patterns match those of breast cancer, with SULF2 being upregulated in the tumor cells [70]. In prostate cancer, overexpression of SULF2 in the transfected prostate cancer cell

lines DU-145 and PC3 presented an oncogenic phenotype, with prostate cancer cells showing greater viability and increased migration capacity [71]. In a study utilizing patient samples with renal cell carcinoma, high SULF2 expression in the tumor cells was correlated with a less invasive phenotype, with low SULF2 expression correlating with advanced invasive features [72]. While the utilization of each SULF by these cancer types remains under investigation, the known ability of these enzymes to modulate growth factor release in the TME makes them interesting enzymes to define. Currently, studies aim to investigate why the two SULF isoforms have variable regulation patterns among various cancer subtypes.

6.5.3 HPSE

Heparan sulfates on perlecan bind a wide variety of molecules in the TME, creating a reservoir of rich growth-promoting and angiogenic factors. Many HBGFs can bind simultaneously to a single heparan sulfate chain depending on its length and pattern of sulfation. As an endo-β-D-glucuronidase, heparanase specificity relies on the O-sulfation along the heparan sulfate chains on the full-length molecule and cleaves the GAG at specific undersulfated regions. These regions typically flank the highly sulfated sites to which most HBGFs are attached [73]. Growth factors such as FGF2/bFGF and VEGF released by HPSE provide an important mechanism supporting neovascularization in cancer, illustrating some of the influence of HPSE-released growth factors on cancer progression [74, 75].

In many cancers, elevated expression of HPSE is associated with poor prognosis, indicating its key role in the promotion of primary tumors to lethal disease [76–78]. HPSE expression in clear cell renal cell carcinomas positively correlates with patient outcomes, with those patients expressing higher HPSE experiencing higher levels of invasion and metastasis [79]. In the case of breast cancer, studies performed with cells overexpressing HPSE showed that tumors grew faster and showed increased vascularization [78]. One study examining prostate cancer clinical samples showed that HPSE levels were significantly higher in cancer tissue than in the corresponding normal tissues that were sampled [80]. In another study increased levels of HPSE were associated with increased metastasis and, in the case of breast cancer metastasis, bone resorption was observed [81]. Because of the correlation between HPSE and cancer aggressiveness, several HPSE inhibitors have been developed and tested in preclinical models with promising results, showing a reduction in tumor growth and reduced angiogenesis [82]. While these inhibitors have shown promising results, current thinking is that efficacy will be most enhanced as a combinatorial therapy. As these inhibitors progress through clinical trials, it will be interesting to see what combinatorial agents are most effective.

6.6 Conclusions, Perspectives, and Future Directions

Emerging evidence places perlecan as a border proteoglycan and signaling hub in the basement membrane and pericellular matrix, where it coordinates and integrates a myriad of cellular signals to maintain proper tissue homeostasis. In cancer, where normal tissue compartments are disrupted by tissue turnover, perlecan becomes a participant in aberrant signaling that fosters progression, invasion, and metastasis. Differences in perlecan's ability to signal depend on context to explain its effects on tumor growth, angiogenesis, blood vessel integrity, endothelial cell proliferation, cancer cell adhesion, and motility [20]. In the context of the TME, cellular behavior can be modulated by the actions of perlecan's modifiers that can change its structure. MMPs, particularly MMP-7, cleave perlecan producing fragments that can have very different bioactivities from the intact proteoglycan [53, 83]. MMP-7 stands out as it demonstrates a unique ability to degrade perlecan without prior removal of the GAG chains in reactive stroma [53]. One key "hot spot" for function-altering cleavage is domain IV, a key region of the core protein that functions to determine cell-cell versus cell-matrix

interactions. Another key functional region of the core protein is evidenced by studies showing that C-terminal cleavage of perlecan produces fragments from domain V that modulate angiogenesis [38, 39, 84]. Thus, while intact perlecan can serve as a suppressor of invasion and angiogenesis, MMP-cleaved perlecan can support cell migration, enhancing tissue turnover, and triggering angiogenesis, a phenomenon that we have called the "molecular switch." This switch can lead to diverse outcomes, either positive or negative, for tumor progression [20].

Other enzyme modifiers in the TME, SULFs and HPSE, work to influence localized bioavailability of HBGFs. Strategies to either restore or reduce sulfatase expression/activity, depending on cancer type, using small molecule inhibitors can help to create novel cancer treatments. HPSE cleaves heparan sulfate chains with associated growth factors. In cancers that preferentially metastasize to bone HPSE is elevated, correlating with poor prognosis [76–78]. One of the HBGFs bound on the heparan sulfate chains that is released is FGF2/bFGF, which participates in neovascularization in cancer [74]. An attractive approach to inhibit HPSE is the use of neutralizing antibodies, though some small molecule compounds have also been investigated [85]. Future work envisions studies of the effects of perlecan fragments on inflammation, recruitment of immune cells to tumor sites, production of circulating tumor cells, and formation of metastases and how the perlecan modifiers can be targeted to prevent cancer dissemination.

The perlecan-rich bone marrow is initially a "hostile" niche. Metastatic cancer cells adapt to this niche and thrive in the TME. Recent studies identify perlecan modification by MMPs and glycosaminoglycanases as main factors to trigger a desmoplastic reaction. In the TME, chronic inflammation and abnormal immune infiltration drive ECM deposition, catabolic enzyme production, and tissue turnover. It is interesting to consider that combining immunotherapy or existing chemotherapies with targeted therapies (e.g., MMP, SULF or HSPE inhibitors, or angiogenesis/inflammation blockers) might lead to new ways to limit bone metastases by stabilizing the tumor-suppressing properties of perlecan in the TME. The main challenge is to identify and tailor the treatment to the individual cancer type, a goal that can only be achieved by first thoroughly understanding the ways that perlecan and its modifiers interact in the context of the TME.

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Heparan Sulfate in the Tumor Microenvironment

7

Barbara Bartolini, Elena Caravà, Ilaria Caon, Arianna Parnigoni, Paola Moretto, Alberto Passi, Davide Vigetti, Manuela Viola, and Evgenia Karousou

Abstract

The biology of tumor cells strictly depends on their microenvironment architecture and composition, which controls the availability of growth factors and signaling molecules. Thus, the network of glycosaminoglycans, proteoglycans, and proteins known as extracellular matrix (ECM) that surrounds the cells plays a central role in the regulation of tumor fate. Heparan sulfate (HS) and heparan sulfate proteoglycans (HSPGs) are highly versatile ECM components that bind and regulate the activity of growth factors, cell membrane receptors, and other ECM molecules. These HS binding partners modulate cell adhesion, motility, and proliferation that are processes altered during tumor progression. Modification in the expression and activity of HS, HSPGs, and the respective metabolic enzymes results unavoidably in alteration of tumor cell microenvironment. In this light, the targeting of HS structure and metabolism is potentially a new tool in the treatment of different cancer types.

Keywords

Heparan sulfate · Tumor · Glycosaminoglycan · Proteoglycan · UDP-sugars · Heparanase · Extracellular matrix · Cancer · Cell growth · Invasion · Adhesion · Metastasis · Inflammation · Angiogenesis · Growth factor

7.1 Introduction

Understanding the biology and behavior of tumor cells is pivotal to develop new therapeutic treatments aiming to control cancer spreading. In recent years, there is an increasing attention on the interplay between cancer cells and the respective surrounding microenvironment where the cells reside, the extracellular matrix (ECM). It is now clear that ECM not only provides mechanical support to the cells, but its complex and dynamic network consisting of glycosaminoglycans (GAGs), proteoglycans (PGs), and other macromolecules (i.e., collagens) is able to modulate cell fate both in physiological and pathological conditions [1, 2].

There are great evidences of the involvement of all the classes of GAGs in the modulation of

Barbara Bartolini and Elena Caravà contributed equally.

B. Bartolini · E. Caravà · I. Caon · A. Parnigoni
P. Moretto · A. Passi · D. Vigetti · M. Viola (✉)
E. Karousou (✉)
Department of Medicine and Surgery, University of Insubria, Varese, Italy
e-mail: Manuela.viola@uninsubria.it; jenny.karousou@uninsubria.it

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development of different cancer cells [3–8], underlying the importance of the knowledge of the biology of these molecules in regulating cell signaling, growth, proliferation, and migration. In this chapter, we focus on the role played by heparan sulfate (HS), the related heparin sulfate PGs (HSPGs), and the enzymes involved in its metabolism, as well as the interaction of HS and HSPGs with other biomolecules and the role on the regulation of the microenvironment and the signaling in cancer cells. These aspects are described in the first three sessions, whereas in the fourth session, we focus our attention on the role of the degrading enzyme heparanase on the tumor progression. Finally, we will discuss the present therapeutic strategies that target HSPGs and heparanase in order to control the development of different cancer types.

7.2 Heparan Sulfate and Heparan Sulfate Proteoglycans: Structure and Biological Roles

Heparin and HS are unbranched closely related GAGs and although they share the same disaccharide unit (glucuronic acid (GlcU)-β1,4-*N*-acetylglucosamine (GlcNAc)-α1,4), some peculiar differences exist. Chemically HS structure is less sulfated than heparin, as the latter is constituted between 75% and 85% of the disaccharide unit 2-sulfated-iduronic acid (IdoA-2S) β1,4 N-sulfated glucosamine (GlcNS) [9]. Moreover, heparin is found only in connective tissue mast cells, whereas HS is a GAG ubiquitously found in the body mostly attached to a core protein, either within ECM, on the cell surfaces, or in secreted granules. HS is likely the highest versatile GAG since it is involved in a plethora of biologic functions spanning from structural organization of tissues, cell proliferation control, cell adhesion and migration, angiogenesis, and infections and inflammation to tumorigenesis [10].

Once the HS chains of variable lengths are produced, they are then subjected to a series of reactions, i.e., epimerization of the C5 of the GlcA to iduronic acid and/or N-deacetylation/N-sulfation of GlcNAc residues and O-sulfation at different position of the sugars (C2 of the hexuronic acids and/or C3 and C6 of glucosamine) [11]. The synthetic enzymes are reported to mature together and form complexes within the ER and Golgi, even though the mechanism of their regulation is yet largely unknown [12]. The sulfate groups confer an overall negative charge at physiological pH, which characterizes the type and nature of the interactions with the ligands.

The modification of the newly synthesized GAG chain takes place in the Golgi apparatus, and the type and degree of epimerization and sulfation is tissue and cell specific [13], being overall homogenous within an organism [14] and dependent on the rate of expression and activity of the different synthesizing enzymes [12, 15, 16]. Specific pattern of iduronization and sulfation within the HS GAG chain has been shown to have relevant physiological effects, and therefore it is highly conserved (binding to growth factors, cytokines, ECM molecules, and triglyceride- or cholesterol-rich lipoprotein, where a decrease in sulfation causes an accumulation of lipid-rich particles) [17].

Moreover, since the polymerization of a GAG chain needs UDP-sugar availability inside the Golgi and ER, the family of nucleotide-sugar transporters (SLC35) consisting of at least 17 molecular species in humans are generally deregulated together with HS synthetic enzymes [18]. The cytoplasmic pool of UDP-sugars is important for the chemical synthesis of HS as well as for its regulation, i.e., for the O-GlcNAcylation post-translational modifications and for the glucuronidation processes, whereas the pool present within Golgi and ER is strictly necessary for the glycoconjugate production. A schematic representation of the overall synthesis and modification of HS chains is shown in Fig. 7.1.

Besides the biosynthetic complexes, the degrading enzymes are also of great importance for the homeostasis of the HS chains. Degradation involves the 6-O-sulfatases, which removes 6-O-sulfate group in glucosamine resi-

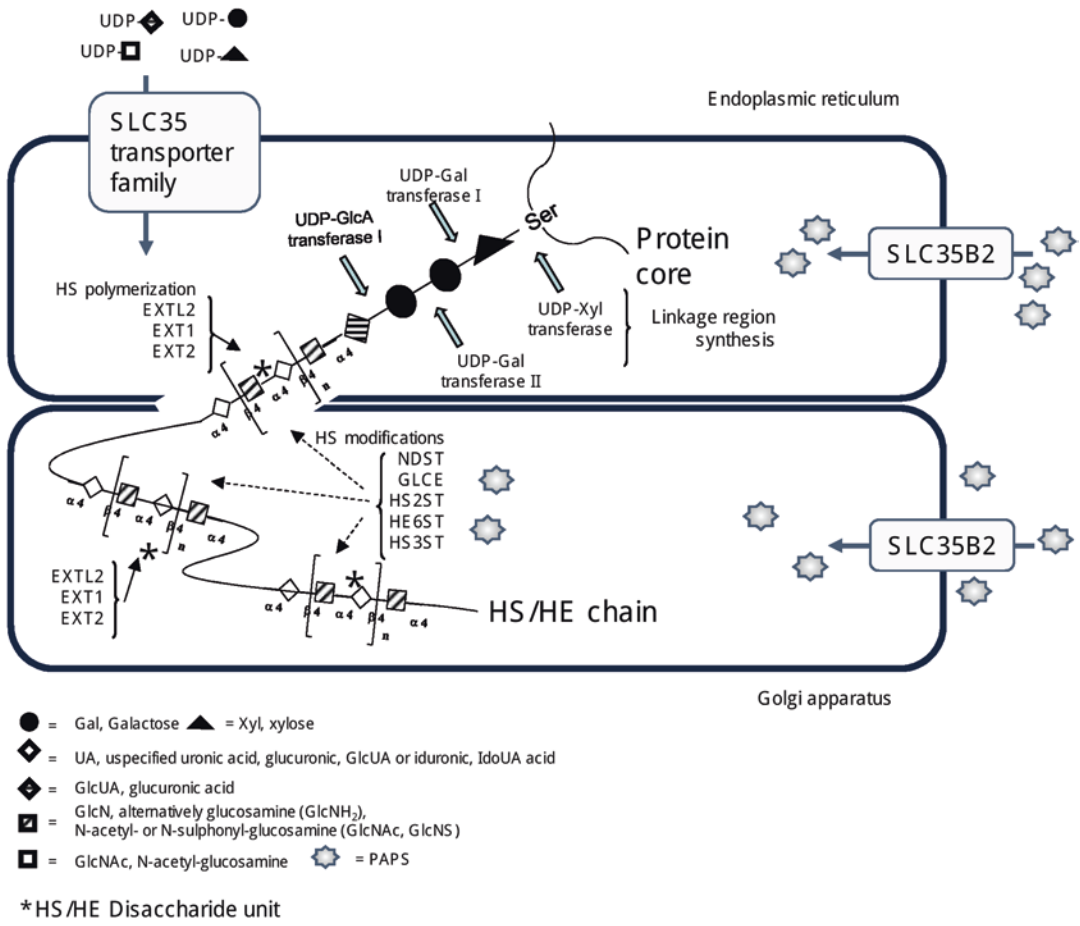

Fig. 7.1 Schematic illustration of HS synthesis. Solute carrier family 35 (SLC35) transport UDP-sugars inside the endoplasmic reticulum, where specific transferases and/or HS-polymerases use them to construct the HS polymer. SLC35B2 member carries phospho-adenosine phosphosulfate (PAPS) within the organelles; PAPS donates the sulfate group for the chain modifications; the enzymes that accelerate the HS polymerization and modification of the disaccharide units are reported with their gene names (HS heparan sulfate, HE heparin)

dues, and the heparanases [19]. The latter are endoglycosidases that catalyze the cleavage of internal GlcAβ1–4 GlcNS linkages in both HS and heparin. The heparanase depolymerizes the chains, resulting in altered recognition of ligands or release of signaling factors that were previously sequestered by the saccharidic moiety. Given the specificity of the recognition pattern of HS to the wide variety of binding partners, it is not surprising that a change in its expression may lead to a dysregulated cell response and perturbation of tissue homeostasis, eventually resulting in cancer transformation.

HS side chains are typical of cell membrane PGs, such as the transmembrane syndecans [20] and the glycosylphosphatidylinositol (GPI)-anchored glypicans [21], and of secreted ECM PGs like perlecan, collagen XVIII, and agrin [11]. A schematic representation of HSPGs localization is shown in Fig. 7.2.

Syndecans are transmembrane PGs with a short C-terminal cytoplasmic domain and an extracellular domain of variable length. The HS chains are usually close to the N-terminus of the extracellular domain [11]. Syndecans are linked to the cell cytoskeleton either directly via the intracellular domain or via an

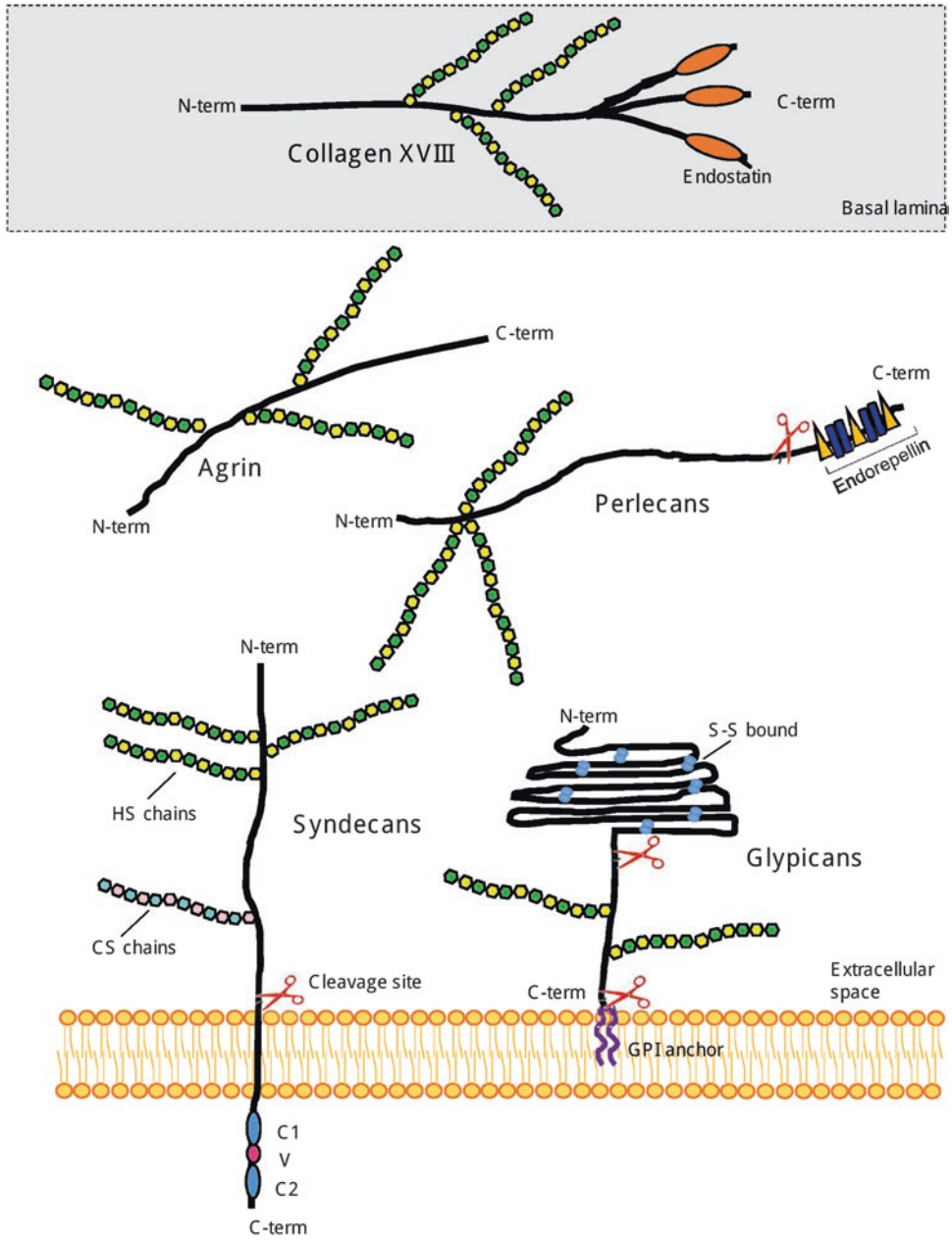

Fig. 7.2 Schematic illustration of cell surface and extracellular HSPGs. Syndecans and glypicans are integral membrane proteoglycans, while perlecans, agrin, and collagen XVIII are extracellular proteoglycans (HS heparan sulfate, CS chondroitin sulfate)

integrin, connecting the ECM to the inner part of cell. These features account for the peculiar roles in cell shape reorganization, adhesion, and motility, which are processes that are usually modified during angiogenesis or migration of tumor cells from the host tissue to new tissues.

Glypican isoforms are largely found in tissues during development and their expression is reduced in the adult. Recent findings showed the

expression of glypican-3 isoform to be critical in solid liver cancer, making the molecule a potential tumor marker and a target for therapeutic treatment [22].

Secreted HSPGs like perlecan, agrin, and collagen XVIII are found in the ECM and in the basement membrane. These PGs sustain the structure of the tissue by interacting with several matrix molecules such as fibronectin and laminin and cell surface receptors like integrins [11, 23].

Perlecan is a major HSPG of basement membranes and of many ECMs of connective tissues. Its HS GAG chains interact with matrix components like laminin, collagen IV, and fibronectin [24]. Moreover, perlecan coordinates cell adhesion, angiogenesis, and thrombosis thanks to its N-terminal HS side chains that serve as a reservoir for vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2) [25]. Mice lacking for the N-terminal HS show defects in corneal angiogenesis [26], underlying the importance of this GAG for the development.

Collagen XVIII is a PG of the basement membranes, bearing three HS attachment sites. Again, the HS side chains are important to recruit growth factors and cytokines at particular sites, as it happens for the monocyte chemotactic protein-1 (MCP-1) that is held in place to be presented to monocytic cells [27].

Agrin was originally identified as organizer of nervous system ECM. In addition, it has been found to play a central role in the niche of lymphoid organs, and it is a component of the basement membrane of blood vessels in several organs [28].

7.3 HSPGs Interaction with Growth Factors

The biological functions of HSPGs are mostly mediated by interactions with proteins called HS-binding proteins (HBP). A wide variety of ligands can interfere with HS including growth factors, cytokines, chemokines, morphogens, matrix proteins, lipoproteins, and various proteins associated with diseases and cancer [29]. Most of the HS ligands are summarized in Table 7.1.

Despite that the interactions may involve the PG core proteins, in most cases the binding requests specific sites of HS chains. In particular, negatively charged groups of HS chains interact with basic amino-acid residues within the protein ligands [30]. This interaction requests the presence of a "consensus sequence" on the ligand protein, characterized by XXBBXBX or XBBBXXBX motif, where B represents basic amino acids and X hydropathic (i.e., neutral or hydrophobic) residues [31]. Nuclear magnetic resonance on the secondary structure of proteins highlighted that the binding can interest also other domains distant from the consensus sequence, such as N-terminal, C-terminal, or region rich in Lys-Asp that offers an appropriate space for the interaction with negative charges of HS chains [32]. The binding with HS chains occurs in a length- and sulfation pattern-dependent manner. The region concerned can be composed by a few disaccharides up to 12-mer [33], and the interactions can interest different sites within the GAG, depending on HBP involved [34]. HS polysaccharide chains are arranged in various types of domains: consecutive N-sulfated disaccharide units (NS-domain), alternated N-acetylated and N-sulfated units (NA/NS domain), and essentially unmodified N-acetylated sequences (NA-domain) [35]. The dissimilar HS composition present in different tissues suggests a high degree of specificity and selectivity in HS/protein interaction [14]. Even if some sulfation patterns seem to be more common than others, the HS region responsible for the interaction contains NS domains in most of the cases. Moreover, despite that functional interactions between HS chains and the respective HBP depend on sulfation density [36], some authors underline that the overall charge is crucial for binding of several proteins, more than the presence of specific HS sulfation sequence [11].

Table 7.1 Heparan sulfate binding proteins

Cell surface	L-Selectin, P-selectin	[92]
	N-CAM (neural cell adhesion molecule)	[93]
	PECAM-1 (platelet endothelial cell adhesion molecule)	[94]
	FGF receptor	[95]
	HIP (heparin/heparan sulfate interaction protein)	[96]
	MAC-1	[94]
Extracellular matrix	Collagens	[97]
	Fibronectin	[30]
	HB-GAM (heparin binding growth-associated molecule)	[98]
	Laminin	[99]
	Tenascin	[100]
	Thrombospondin I and II	[101]
	Vitronectin	[102]
Growth factors	HB-EGF (heparin binding epidermal growth factor)	[103]
	FGF (fibroblast growth factor)	[104]
	VEFG (vascular endothelial growth factor)	[105]
	HDGF (hepatoma-derived growth factor)	[106]
	P1GF (placenta growth factor)	[107]
	PDGF (platelet-derived growth factor)	[97]
	TGF-β (transforming growth factor)	[97]
	HGF (hepatocyte growth factor)	[108]
Cytokines/chemokines/ morphogens	BMP (bone morphogenic protein)	[109]
	IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12 (interleukins)	[110]
	IP-10 (interferon-γ-inducible protein)	[111]
	CCL-2 (CC-chemokine ligand)	[112]
	GM-CSF (granulocyte macrophage colony-stimulating factor)	[113]
	MCP-1, MCP-2, MCP-3, MCP-4 (monocyte chemoattractant protein)	[112, 114]
	RANTES (regulated on activation, normal T cell expressed and secreted)	[112]
	TNF-α (tumor necrosis factor)	[111]
	MIP-1α, MIP-1β, MIP-2 (macrophage inflammatory proteins)	[115, 116]
	PF-4 (platelet factor)	[117]
	Hh (Sonic Hedgehog)	[109]
	Wnt (Wingless wg)	[109]
Others	DNA and RNA polymerases	[118]
	Superoxide dismutase	[119]
	Angiogenin	[120]
	Cathepsins B and G	[121]
	Neutrophil elastase	[122]
	Annexin V	[123]
	Prion	[124]
	β-Amyloid protein	[125]
	Na^+/Ca^+ exchanger protein	[126]
	Myosin ATPase	[127]

Table modified from Dreyfuss et al. [128]

7.4 HS-Growth Factors Interplay and Cancer

The biosynthetic reactions and the post-synthetic modification that occur on the HS chain (Fig. 7.1) must be carefully regulated within the cell, to generate a fully functional GAG. Several reports prove that an aberrant functionality or a deregulation of either biosynthetic or catabolic enzymes results in a modified chain that fails to support the physiological properties of HS [11, 37]. An alteration of HS structure, and therefore function, can interfere with cell homeostasis enhancing or inhibiting cell growth, prominently because of the modification of the interaction between HS and its ligands. A modification of the pattern of sulfation may change the recognition site for specific growth factors, causing a sustained activity of the ligand or a lack of signal. Indeed, there are several distinct mechanisms through which HSPGs regulate growth factor activity in the surrounding microenvironment, such as extending the half-life of the ligand, controlling its diffusion, and modulating the interaction with its tyrosine kinase receptor [29, 38].

Cell surface PGs are the most reported to be involved in tumor onset and cancer progression. Among them, the family of syndecans (SDC-1, SDC-2, SDC-3, SDC-4) are the principal HSPGs described in the tumor microenvironment that are recognized to be key macromolecules in the progression of various types of cancer.

Many studies highlight the role of syndecan-1 in various human cancers [29]. Syndecan-1 acts as a co-receptor for different growth factors (such as FGF2, VEGF, Wnt, HGF, and IL-8) and their receptors, stabilizing the growth factor receptor complexes followed by activation of downstream tyrosine kinase pathways [39, 40], through its intracellular domain. In particular, syndecan-1/FGF2 interaction promotes angiogenesis, proliferation, dysregulated growth, and tumor progression in lymphomas, breast cancer, and prostate cancer [41–43]. Moreover, syndecan-1 is upregulated in multiple myeloma, and its interaction with VEGF, an important regulator of angiogenesis, is actually implicated in the pathogenesis of the disease [44, 45].

The members of the other family of membrane HSPGs, glypicans (glypican-1, glypican-3), interact with growth factors, cytokines, morphogens, and enzymes, also leading to tumor growth and invasion. Loss-of-function mutation in glypican-3 causes the human X-linked disorder Simpson-Golabi-Behmel syndrome and prepares to certain pediatric solid embryonal tumors, including Wilms tumor, hepatoblastoma, adrenal neuroblastoma, gonadoblastoma, hepatocellular carcinoma, and medulloblastoma [46]. An overexpression of HSPGs like glypicans has been detected in malignant breast cancer tissue [47]. In these circumstances, HS enhances the presentation of FGF to its receptor, leading to prominent activation of the tyrosine kinase downstream activity [48]. Similarly, the persistent activation of proliferation signaling has been shown in other types of cancer, such as pancreatic cancer [49], gliomas [50], and hepatocellular carcinoma [51].

Although secreted HSPGs apparently fulfill the maintenance of a correct architecture of the ECM, a change in their HS structure has been linked not only to structural changes but also to cancer transformation.

Perlecan's ability to regulate angiogenesis through interaction with VEGF and FGF2 makes it a central modulator of possible new vessel formation in the setting of tumor growth and invasion enhancing metastatic potential [25]. Indeed, perlecan is expressed in a lot of tumors such as human salivary adenoid cystic carcinoma, ameloblastomas, and liver carcinoma [52].

Collagen XVIII has recently attracted interest because its endostatin domain has an inhibitory role in angiogenesis, lymphangiogenesis, and cancer metastasis [53]. Moreover, elevated levels of endostatin in tissue and in blood are associated with a long-term survival in human hepatocellular carcinoma [54].

Overexpression or downregulation of collagen XVIII has been observed in different tumors. For instance, low expression of collagen XVIII in human hepatocellular carcinoma is associated with tumor malignancy [55]. On the other hand, collagen XVIII is upregulated in many tumors such as invasive breast cancer, cutaneous and oral

squamous cell carcinoma, lung cancer, pancreatic cancer, colorectal cancer, and ovarian cancer [56].

Lastly, high levels of expression of agrin are related to hepatocellular carcinoma, where it promotes cell proliferation, migration, and epithelial-to-mesenchymal transition [57]. The spatial reorganization of overexpressed agrin modifies the permeability of vascular basement membrane [58], thus contributing to modulate the diffusion and accumulation of metabolites in the tumor microenvironment which, in turn, will influence tumor cell behavior.

Interestingly, HS interaction with Hedgehog proteins regulates their ability to form morphogen gradients during organogenesis. For the vertebrate, Hedgehog proteins (a family of morphogens that include Sonic (Shh), Indian (Ihh), and Desert (Dhh) Hedgehog) are important for embryonic development, and their distribution on target cells has been shown to be regulated by HS and HSPG interaction [59]. The dysregulation of this pathway is associated with the development of several types of cancer. Notably, the Shh signaling is often upregulated in pancreatic cancer and HS binding activity of Shh is required for its action on the proliferation and metastatic spreading of pancreatic ductal adenocarcinoma cells [32, 60].

It is noteworthy that in the progression of cancer, the immune system can be misled and ignore the tumor growth or even contribute to its growth [61]. In breast cancer for example, the HSPG syndecan-1 has been suggested both as a regulator of cancer stem cell (CSC) phenotype and as a modulator of lymphocytes, in particular of T helper cells (Th cells) within the tumor microenvironment depending on the subtype of the disease (non-inflammatory breast cancer vs. IBC) [62].

7.5 Heparanase Contribution to Tumor Progression

Beyond the structural modification of the HS structure and pattern sulfation, a variation of its total amount may alter the stiffness of the ECM, thus modulating cell adhesion and migration. A stiff ECM does not provide room for preferential cell migration, preventing or delaying cell movement and so metastasis spreading. Reorganization of the PGs and ECM may disclose new binding partner(s) in the tumor stroma and attract cancer cells, starting the metastatic transformation. The mechanisms underlying this process are complex and not fully understood. Several enzymes are involved in the matrix remodeling and the downregulation or inhibition of one of them may imply the activation of a parallel pathway [18].

In this light, a central contribution comes from the action of an HS-degrading enzyme, the heparanase. This enzyme is recruited to cell surface by binding to HSPG side chains or to other receptors like the lipoprotein receptor-related protein (LRP) and the cation-independent mannose-6-phosphate receptor (CIMPR) [63, 64]. Heparanase is then internalized and activated within the cell.

Heparanase can be active as tumor inducer also independently of its enzymatic activity. In fact, it has been shown to activate several signaling pathways involved in angiogenesis, cell proliferation, and migration, through Akt, STAT, Src, Erk, HGF-receptor, IGF-receptor, and EGF-receptor signaling. Together with the signaling stimulation, heparanase is able to stimulate the transcription of genes involved in the aforementioned pathways (i.e., angiogenesis, proliferation, inflammation) [65–67]. Tumor cells with elevated expression of this enzyme are therefore able to modify the fate of surrounding cells and to promote the progression of tumor itself in a positive-feedback loop. Indeed high level of heparanase has been strictly related to poor prognosis and increased aggressiveness of several cancer types [68–70], and patients who after surgery have less active heparanase had better prognosis and longer survival [70].

Moreover, cytokines and growth factors bound to HSPGs can be mobilized by cleavage of HS chains catalyzed by heparanase. In human cancer, an abnormal high shedding of syndecan-1 and syndecan-2 from cell surface corre-

lates with metastatic potential, tumor vascularity, and reduced postoperative survival of cancer patients [71, 72]. The mechanism of the proteoglycan shedding is mediated in tumor by several metzincin enzymes (such as metalloproteinases) which cleave the PG ectodomain near the plasma membrane. These processes together with the action of heparanase deeply and extensively change the function of the HSPGs in the tumor microenvironment, giving them an alternative role to the one carried out in the physiological tissue [73].

Another intriguing feature of the heparanase is its involvement in the regulation of inflammation and immune response. Since among HS binding partners there are a number of cytokines, the disruption of the binding can potentially start immune responses [74] that may attract immune cells at the site of the tumor. Immune cells can then contribute to the alteration of the microenvironment through the release of their granules and secretion of other chemokines and cytokines. Recently it has been discovered that human in vitro-cultured chimeric antigen receptor (CAR) T cells lack expression of the enzyme heparinase [75], and this could result in a higher antitumor activity of the CAR-redirected T cells.

7.6 Targeting HS: Potential Therapeutic Agents in Tumor

Considering the ubiquitous presence of HSPGs in tissue and the multiple roles played in the initiation and amplification of tumor transformation, it appears crystal clear how the targeting of HS could disclose huge potentiality for cancer treatment. Interfering with the expression or with the activity of heparanase will result in a modified HS and therefore a modified ECM and tumor microenvironment. Inhibition of heparanase is likely a promising target for the inhibition of both angiogenesis and metastasis [76]. Several studies performed on mice have demonstrated the potential of knocking down heparanase in the outcome of tumor treatment [77–79]. Of great interest is the observation that inactive heparanase is anyway able to induce VEGF and HGF expression and the related downstream signaling pathways [80]; therefore, a correct approach should take into consideration the inhibition of the growth factor binding site as more than a mere inactivation of the enzyme. In this light, the resolution of the crystal structure of the heparanase will help to shed light on drug design [81], both in terms of inhibiting peptides and specific antibodies.

An interesting observation is that heparin, thanks to its structural relation to HS, is a natural inhibitor of heparanase. Notwithstanding, its wide use as anticancer agent is restricted by the anticoagulant activity. Several studies have analyzed the use of low-molecular-weight heparin in cancer therapy with contradictory results [82–85]. Other strategies imply the use of oligonucleotides that act as heparanase inhibitors by reducing its expression together with a series of synthetic inhibitors, exhaustively reviewed in [86].

In addition to heparanase, another possible point of intervention could be the targeting of the endostatin domain of collagen XVIII. Many studies convincingly demonstrated that endostatin exerts an efficient inhibitory effect on tumor angiogenesis and growth in animal models [87]. Consequently, endostatin's antitumoral properties make it a very attractive therapeutic agent in the treatment of cancers, and several II phase clinical trials are using the recombinant endostatin (alone or in combination with other chemotherapy drugs) as antitumoral growth and anti-angiogenesis agent [88, 89].

The development of synthetic peptides that specifically interfere with the interaction between HS and its binding partners is also an attractive alternative. This strategy could theoretically allow modulating singularly the interaction with the growth factors, acting only on specific pathways. The study of Dogra et al. reported encouraging results [90] and the possibility to interfere with the HS in the tumor microenvironment is a field to be explored.

7.7 Conclusions and Future Perspectives

Both HS and HSPGs can exert a number of functions within tumors as they have the ability to promote or inhibit the initiation and progression of various tumor types. The role of HS/HSPGs is highly associated with specific domains within the structure of the HS chain and the proteoglycan cores, as well as the localization of HSPG in ECM or cell surface. Several studies are focused on the precise molecular mechanism by which HS and HSPGs interact with other cellular proteins, such as growth factors, regulating specific signaling pathways in various tissues. Similarly, heparanase biology that includes enzymatic and non-enzymatic activity was proved to play a pivotal role during tumor progression, rendering this enzyme a great target for the development of heparanase inhibitors that could be applied to treat not only cancer but also other diseases [91]. Thus, deciphering the molecular role through which HS/HSPGs and heparanase modulate the tumor-stroma microenvironment that favors tumor growth and metastasis could be an attractive therapeutic target.

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8 The Role of Glypican-1 in the Tumour Microenvironment

Maria E. Lund, Douglas H. Campbell, and Bradley J. Walsh

Abstract

Glypican-1 (GPC-1) is a cell surface heparan sulphate proteoglycan that is critical during normal development, but which is not required for normal homoeostasis in the adult. It is, however, overexpressed in a variety of solid tumours and is known to regulate tumour growth, invasion, metastasis and progression, through modulation of tumour cell biology as well as influence on the tumour microenvironment (TME). The role of GPC-1 in the TME and on the tumour cell is broad, as GPC-1 regulates signalling by several growth factors, including FGF, HGF, TGF-β, Wnt and Hedgehog (Hh). Signalling via these pathways promotes tumour growth and invasive and metastatic ability (drives epithelial-to-mesenchymal transition (EMT)) and influences angiogenesis, affecting both tumour and stromal cells. Broad modulation of the TME via inhibition of GPC-1 may represent a novel therapeutic strategy for inhibition of tumour progression. Here, we discuss the complex role of GPC-1 in tumour cells and the TME, with discussion of potential therapeutic targeting strategies.

M. E. Lund (✉) · D. H. Campbell · B. J. Walsh
GlyTherix Ltd, Sydney, NSW, Australia
e-mail: Maria.lund@glytherix.com; Douglas.campbell@glytherix.com; Brad.walsh@glytherix.com

Keywords

Glypican-1 · GPC-1 · Growth factor signalling · Therapeutics · Pancreatic cancer · Tumour microenvironment · Metastasis · Angiogenesis · Invasion · Stroma

8.1 Introduction

The survival, growth and metastasis of a mass of tumour cells relies on its successful interaction with the collection of resident cells such as cancer-associated fibroblasts (CAFs) and infiltrating host cells such as immune cells including macrophages, cytokines, growth factors, blood vessels and extracellular matrix proteins. These are collectively known as the tumour microenvironment (TME). From a therapeutic perspective, tumour control may be achieved by manipulation of this interaction, by targeting elements of the TME crucial to tumour growth and/or survival. Understanding the complex interplay between tumour and TME will help in the identification of the most promising therapeutic targets.

Glypican-1 (GPC-1) is a cell surface heparan sulphate proteoglycan that is overexpressed in a variety of solid tumours, as well as during development, but whose expression is suppressed in most adult normal tissues [1, 2]. Glypican-1 is known to play a critical role in the biology of the

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tumour cell, being involved in cell cycle and proliferation, as well as invasive and metastatic capability [3, 4]. This review will focus on the complex role of GPC-1 in the solid tumour microenvironment and how its interaction with growth factors in the TME influences tumour growth, invasion and metastasis as well as CAF biology and angiogenesis. We will consider the role of GPC-1 in establishment and maintenance of cellular components of the stroma, with a special focus on the role of GPC-1 in pancreatic cancer. Finally, we will discuss potential therapeutic strategies for targeting of GPC-1 in the TME.

8.2 Glypican-1

Glypican-1 belongs to the family of glypicans (1–6), all of which are anchored to the cell surface by glycosylphosphatidylinositol. The heparan sulphate chains are covalently linked to the core protein, and these chains, when anchored to a cell, allow glypicans the ability to sequester and retain growth factors from the environment close to signalling molecules on the cell surface, facilitating the initiation and perpetuation of cell signalling [5]. In this respect, GPC-1 acts as a form of co-receptor for a range of signalling molecules, influencing signalling pathways including Wnt, Hedgehog (Hh), TGF-β and fibroblast growth factor (FGF) [6].

Glypican-1 is normally expressed during embryonic development in a temporally and spatially regulated manner. Animal studies have revealed a requirement for GPC-1 in normal brain development [7], and it is expressed in the developing skeletal system, bone marrow and kidneys; however, it is not required for normal homoeostasis [8]. Reports examining expression in normal adult tissue by immunohistochemistry have differed somewhat in reported expression, potentially attributable to the use of different antibodies; however, collectively, these studies show that GPC-1 expression is not observed in most normal adult tissues [1, 2].

Glypican-1 is known to promote tumour growth, metastasis and invasion, as detailed in this review and elsewhere [9]. In line with this, expression of GPC-1 has been described in a variety of solid tumours. Expression of GPC-1 was seen in 80% of prostate tumours, with no expression in benign prostate biopsy tissue [1]. Expression has been demonstrated in pancreatic cancer specimens but not normal pancreata [10]. Immunohistochemistry of normal pancreata (n = 169), pancreatic adenocarcinoma tissue (n = 186) and metastatic tumours in the liver (n = 4) revealed GPC-1 expression in tumour tissue but not in normal tissue [11]. Glypican-1 expression was seen in breast cancer by IHC and confirmed by in situ hybridisation, but not in normal tissue [4]. Significantly higher GPC-1 expression was observed in glioma specimens (astrocytomas n = 49 and oligodendrogliomas n = 7) than in non-malignant tissue [12]. Another study demonstrated expression in 27 of 53 surgically resected glioblastoma samples [13]. Expression in oesophageal squamous cell carcinoma (ESCC) was observed in 98.8% of specimens [14]. Expression of GPC-1 has been described in cervical cancer (adenocarcinoma and squamous cell carcinoma) [15] and in epithelioid mesothelioma in two studies [16, 17]. Interestingly, the study by Amatya and colleagues (2018) showed that expression of GPC-1 could differentiate epithelioid mesothelioma from lung adenocarcinoma with almost 100% sensitivity and 97% specificity, whilst the work described by Chiu et al. (2018) showed no differentiation between the two malignancies as most specimens for both indications stained positive for GPC-1.

Our understanding of the role of GPC-1 in tumour invasion and metastasis would predict the link between high tumour GPC-1 expression and poor clinical prognosis. For example, high GPC-1 expression in ESCC tumours, as measured by IHC, was associated with worse clinical outcomes than those tumours expressing low levels of GPC-1, potentially related to a relationship between GPC-1 expression and chemoresistance [14]. In glioblastoma, patients whose tumours stained positive for GPC-1 had a shorter overall survival than those whose tumours were negative [13]. In pancreatic ductal adenocarcinoma, higher GPC-1 levels in the tumour were associated with worse tumour biological features,

including worse pathological differentiation and larger tumour sizes [11]. In line with these observations, patients whose tumours expressed GPC-1 had a shorter overall survival time.

Preclinical studies provide significant insight into the critical role of GPC-1 in tumour cell growth, invasion and metastasis in a variety of solid tumours. Indeed, GPC-1 is physiologically necessary for signalling via some mitogenic pathways that are required for tumour cell proliferation, characterised for breast and pancreatic cancers [4, 18]. For example, reduction of GPC-1 expression by transfection of Colo-357 pancreatic cancer cells with an anti-sense construct reduced anchorage-dependent and anchorage-independent cell growth [19]. PANC-1 cells in which GPC-1 was knocked down showed inhibited cell growth in vitro (longer doubling times and reduced anchorage-independent cell growth) and were less able to form tumours in vivo in immune-deficient mice, additionally demonstrating reduced angiogenesis and metastasis associated with GPC-1 knockdown [3]. Interesting studies into the role of glycosaminoglycans (GAGs) as mechanosensors for interstitial flow on cancer cells have helped elucidate the role of GPC-1 in metastasis, as knockdown of GPC-1 in a metastatic renal carcinoma cell line SN12L1 completely inhibited migration, with the GPC-1 core protein acting as a link between the GAG and cell, promoting metastasis [20]. Glypican-1 knockdown inhibited tumour growth in a KrasG12D-driven genetic pancreatic cancer model, affecting angiogenesis and metastasis [21]. In ESCC, blocking GPC-1 with an antibody inhibits tumour growth in a mouse PDX model [2].

Importantly, GPC-1 expression is not just required in the tumour cell for effective tumour growth, as GPC-1^{null} mice exhibited defects in tumour angiogenesis and metastasis in an orthotopic xenograft model of pancreatic cancer, where the tumour cells were wild type for GPC-1 (control tumour cells) [3]. This points to a critical role for GPC-1 not only in the tumour cell but also in the TME, in both angiogenesis and the modulation of resident (host) cell function.

Here, we examine how GPC-1 interacts with and influences elements of the TME to control tumour cell survival, proliferation, invasion and metastasis.

8.3 Glypican-1 in the Tumour Microenvironment

Growth factors are critical components of the complex network of chemical mediators that may be derived from host or tumour cells and act in a paracrine or autocrine manner to activate signalling pathways that influence tumour growth, proliferation, invasion and metastasis in both tumour cells and host cells of the TME. Here we discuss evidence for the interaction of GPC-1 with factors, such as Wnts, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), TGF-β and VEGF-A, and the influence of this interaction on cellular elements of the stroma, angiogenesis and tumour cell growth, proliferation, invasion and metastasis. Growth factors influence stromal cells (including CAFs which in turn provide growth factors to the TME), which then influence tumour drug resistance, metastasis, and proliferation and affect angiogenesis. Thus, GPC-1 exerts control over various pathways associated with tumour progression.

8.4 GPC-1 Enhances Growth Factor Signalling to Promote Proliferation, Invasion and Metastatic Potential

The Wnt family of proteins trigger canonical Wnt signalling by binding to FRIZZLED receptors on the cell surface, which inhibits degradation of β-catenin, triggering the translocation of β-catenin to the nucleus and accumulation there, where it regulates the expression of Wnt target genes. Wnt signalling in homoeostasis is involved in cell differentiation, self-renewal and cell migration but is also known to be involved in the establishment and progression of solid tumours (reviewed in [22], including colorectal [23],

prostate [24], breast cancer [25, 26] and melanoma [27]).

The glypicans are known to interact with Wnts [28, 29]. Glypican-3 has been shown to promote canonical Wnt signalling and, hence, hepatocellular carcinoma growth, by two mechanisms: firstly, by binding Wnt and promoting the accumulation of Wnts at the cell membrane, in contact with FRIZZLED, and, secondly, by interacting with FRIZZLED itself, to promote the formation of signalling complexes of Wnt and FRIZZLED [30]. Studies of the interaction between GPC-1 and Wnts are limited, with just one study examining the relationship between Wnt signalling and GPC-1 expression in the forming of trigeminal ganglions of chickens, showing that overexpression of GPC-1 actually phenocopies inhibition of Wnt signalling, thus suggesting a negative regulatory role for GPC-1 (although this is in normal development) [31]. Further investigations into the role of GPC-1 in Wnt signalling in the TME are required to understand this relationship and its implication in tumour biology. Indeed, it is foreseeable that GPC-1 would be involved in Wnt signalling in tumour cells, as GPC-1 is overexpressed in cancers where Wnt signalling is also implicated in tumour establishment/progression, for example, in prostate and breast cancers.

The family of fibroblast growth factors (FGFs) consist of secreted signalling proteins that are expressed in almost all tissues and serve a critical role in embryonic development, organogenesis, and, in the adult, maintenance of homoeostasis with roles in metabolism and tissue maintenance and repair. Signalling involves binding of FGF to FGFR and a heparin sulphate [32]. In the TME, FGFs have broad biological function, influencing angiogenesis, tumour cell migration and invasion and cross-talk between epithelial and stromal cells (reviewed in [32]).

Glypican-1 is known to act as a co-receptor for FGF-2 and heparin binding epidermal growth factor (HB-EGF) [7, 33], influencing the mitogenic response of tumour cells and thus tumour growth. It is thought that the heparan sulphate chains interact with FGF to stabilise the FGF-FGFR complex on the cell surface and/or retain FGF in close proximity to FGFR to encourage signalling [34, 35]. Cleavage of the heparin sulphate chains of GPC-1 in two breast cancer cell lines MDA-MB-231 and MDA-MB-468 arrested the mitogenic response to FGF2 and HB-EGF [4]. Moreover, reducing GPC-1 protein expression by transfection with a GPC-1 anti-sense construct reduced the mitogenic response to both growth factors. Reduction of GPC-1 expression either by enzymatic cleavage of the cell surface expressed GPC-1 or by transfection with an anti-sense construct, in two pancreatic cell lines, resulted in suppression of mitogenic responses to FGF2 and HB-EGF [18]. In ESCC cells, phosphorylation of EGFR was reduced in cells transfected with GPC-1 siRNA following stimulation with HB-EGF [2]. In vivo, treatment of NOD/SCID mice (lacking functional NK cells and CDC) with an antibody that recognises GPC-1 inhibited the growth of an ESCC PDX, in a complement-dependent cytotoxicity (CDC)-independent and antibody-dependent cellular cytotoxicity (ADCC)-independent manner, potentially attributable to inhibition of mitogenic response, although this mechanism was not investigated [2].

There is extensive evidence for the role of both Wnt and FGF signalling in the promotion of cancer EMT, invasion and metastasis [36–38]. High levels of Wnt signalling are associated with a more aggressive phenotype of cancer, for example, in breast cancer [25]. In young prostate cancer patients (<50 years of age), an age group associated with more aggressive cancer, Wnt signalling is higher, and co-expression of β-catenin and androgen receptor (AR) correlates with higher Gleason scores [39]. In metastatic castration-resistant prostate cancer (mCRPC), mutations in the Wnt pathway are more common [40], and those men who have Wnt pathway mutations are less responsive to abiraterone/enzalutamide therapy [41]. Indeed, prostate cancer cells themselves secrete Wnts which act in an autocrine manner to promote tumour progression, but also act on the epithelium [24]. In line with the role of GPC-1 in Wnt and FGF signalling pathways, there is both experimental and clinical evidence for a role of GPC-1 in tumour

invasiveness, as high GPC-1 expression in pancreatic cancer is associated with perineural invasion [10]. Loss of GPC-1 in a GPC-1^{null}mouse model of KrasG12D-driven pancreatic cancer inhibited spontaneous pancreatic tumour invasiveness into surrounding tissue [21]. Pancreatic cancer cells isolated from the tumours of GPC-1^{null}KrasG12D mice were less invasive when stimulated with FGF-2 ex vivo, as compared to tumours from GPC-1$^{+/+}$ mice, and when these cancer cells were then grafted back into mice as tumours, they were less metastatic than tumours from GPC-1$^{+/+}$ mice.

TGF-β binds to cell surface kinase receptors that phosphorylate cytoplasmic Smad proteins, which in turn interact with Smad4 proteins, then translocating to the nucleus to drive gene expression. The role of TGF-β signalling in cancer is an interesting one, as TGF-β is not only known to inhibit tumour growth but also to promote tumour invasion in later-stage disease [42]. Interestingly, once established, tumour cells lose the ability to be growth inhibited by TGF-β, but retention of semi-functional TGF-β signalling favours a more aggressive phenotype [42]. Indeed, a loss of TGF-β signalling is thought to be critical to the pathogenesis of pancreatic cancer [43]. On the other hand, TGF-β ligands, which are commonly seen to be overexpressed in pancreatic cancer, can drive EMT and an invasive phenotype of tumour cell [44, 45]. Studies in a cell line of pancreatic cancer have demonstrated that GPC-1 is involved in TGF-β signalling, as knockdown of GPC-1 resulted in an insensitivity to TGF-β-mediated growth inhibition [19], suggesting a role for GPC-1 in tumour progression through modulation of TGF-β signalling.

It is thought that the Wnt and TGF-β signalling pathways act synergistically to regulate gene transcription involved in tumourigenesis [46]. Interestingly, gene expression of GPC-1 is upregulated when tumour cells are stimulated with Wnt and TGF-β in combination, but not when cells are stimulated with either ligand alone, implying the involvement of GPC-1 in the Wnt/TGF-β collaboration [46].

Cancer stem cells (CSCs) are a subpopulation of tumour cells that are "stem-like" in phenotype, with capacity for self-renewal and differentiation, and that are resistant to chemotherapy and radiation, acting as a pool of cancer cells that can drive recurrence after therapy [47]. This population is thought to be involved in establishment of metastases [48]. Cancer stem cells are described in several solid tumours, including pancreatic cancer, where they are controlled by TGF-β, signalling via which is required for self-renewal and EMT, influencing subsequent invasion. Incubation of pancreatic CSCs with TGF-β drives a mesenchymal phenotype (in morphology and gene expression), and exposed cells display increased invasion in a Matrigel assay [49]. The expression of GPC-1 in CSCs is not yet described and is an area worthy of further research.

8.5 GPC-1 in Angiogenesis

Promotion of growth factor signalling by GPC-1 influences angiogenesis, a process necessary for tumour growth and metastasis. FGF-2 acts as a pro-angiogenic signal to endothelial cells, binding FGFR1 [50]. Glypican-1 is postulated to act as a co-receptor for FGF, encouraging interaction with FGFR1. GPC-1 also interacts with VEGF-A (a potent mitogen for endothelial cells), enhancing signalling through the VEGF receptor. Indeed, expression of GPC-1 has been demonstrated in endothelial cells from glioma samples, but not endothelial cells from normal brain [51]. Wnt signalling, known to be regulated by GPC-1, is critically involved in angiogenesis, through regulation of VEGF transcription [52].

Functionally, knockdown of GPC-1 inhibits angiogenesis in vivo in mice in a human pancreatic cancer xenograft model [3]. In a GPC-1^{null} mouse model of KrasG12D-driven pancreatic cancer, angiogenesis was inhibited and this was associated with smaller tumours [21]. This suppression in angiogenesis was associated with a reduction in mRNA expression of various pro-angiogenic factors, including VEGF, in the tumour. Moreover, tumours from GPC-1^{null} mice expressed less CD34, a marker of angiogenesis. Endothelial cells isolated from GPC-1^{null} mice were non-migratory in response to VEGF-A,

evidence for a role for GPC-1 in establishment of angiogenesis. In a mouse model, treatment of mice bearing ESCC PDXs with an anti-GPC-1antibody (reactive to mouse GPC-1) inhibited tumour growth, and this inhibition was independent of antibody effector functions (ADCC and CDC) [2]. Instead, the authors described expression of GPC-1 in the vascular endothelium of the tumours and postulated that the reduction in tumour growth associated with anti-GPC-1 antibody treatment may be attributed to inhibition of angiogenesis. Indeed, treatment with the anti-GPC-1 antibody, as compared to an isotype control antibody, was associated with a decrease in the concentration of blood vessels (identified by CD31 positivity) in the tumour [2].

8.5.1 The Influence of GPC-1 on Stromal Cells: Highlight on Pancreatic Cancer

Pancreatic cancer is a highly lethal malignancy. Survival rates are dismal with the 5-year survival rate at just 17%. Normally diagnosed at late stage, the standard of care is gemcitabine after surgical resection which provides only a marginal survival benefit [53]. The complex interaction between the stroma and the tumour cells themselves must be understood to develop novel therapeutic approaches.

A role for GPC-1 in pancreatic cancer is well established, and clinical evidence demonstrates that high expression is associated with perineural invasion and associated poor prognosis [10, 18]. Two pertinent studies examined carefully the role of GPC-1 in pancreatic cancer initiation and progression, establishing a critical role for GPC-1 in both the pancreatic tumour cell and the host TME. The study by Aikawa and colleagues (2008) [3] showed that knockdown of GPC-1 in the human pancreatic cell lines PANC-1 and T3M4 using anti-sense constructs inhibited tumour growth, metastasis and angiogenesis when engrafted in mice in an orthotopic xenograft. Importantly, the same study demonstrated a requirement for GPC-1 in the host TME for efficient tumour progression, as reduced tumour angiogenesis and metastasis was observed after xeno-engraftment of human pancreatic cell lines into athymic nude mice on a GPC-1^{null} background. The other pertinent study on the role of GPC-1 in pancreatic cancer progression was that by Whipple et al. (2012) [21]. In this study, a genetic mouse model of pancreatic ductal adenocarcinoma (PDAC) was established by pancreas-specific activation of the oncogene $Kras^{G12D}$and loss of pancreatic INK4A expression. This background was then combined with wild-type GPC-1 or GPC-1^{null}. Only 1/10 GPC-1^{null} mice developed tumours, as compared to 7/10 GPC-$1^{+/+}$ mice, and at later-stage disease, tumours from GPC-1^{null} mice were less invasive, as all GPC-$1^{+/+}$ mice (14/14) had large, invasive tumours, whilst just 4/20 GPC-1^{null} mice did. In line with these findings, levels of Ki67, a marker of proliferation, and CD34, a marker of angiogenesis, were reduced in the tumours of GPC-1^{null} mice as compared to those of GPC-$1^{+/+}$ mice. Tumours cultured ex vivo from GPC-$1^{+/+}$ and GPC-1^{null}mice grew more slowly in vitro, were less invasive in response to FGF stimulation and, when tumour fragments were engrafted orthotopically into athymic nude mice, were unable to metastasise. These tumour fragments consisted tumour cells as well as endothelial cells and CAFs, which are involved in metastasis, pointing to a role for GPC-1 in controlling PDAC progression through modulation of both tumour cell and stroma. Here, we discuss the influence of GPC-1 on CAF biology, an important component of the stroma in PDAC.

In pancreatic cancer, CAFs largely arise from pancreatic stellate cells (PSCs) and mesenchymal stem cells (MSCs). The complex interplay between CAFs and tumour cells promotes growth, proliferation and invasion of the cancer cells, whilst CAFs also modify the stromal composition to ensure tumour survival and facilitate metastasis. CAFs secrete a variety of growth factors into the TME, including cytokines, growth factors and exosomes containing miRNAs, whose paracrine actions influence tumour cell function, including driving EMT and invasive ability [54]. This role of CAFs in promoting tumour progression is not limited to pancreatic cancer, but it has

also been shown in breast, colorectal and bladder cancers [55–58]. One of the major molecules secreted by CAFs that drives invasion and EMT in tumour cells is TGF-β, a molecule that plays a complex role in the pathogenesis of pancreatic cancer, suppressing early-stage disease, whilst driving invasion, promoting metastasis and angiogenesis and contributing to immune evasion in later stages [59–61]. Glypican-1 is known to modulate the TGF-β signalling pathway in pancreatic tumour cells, potentially playing a role in tumour cell proliferation [19, 62]. Importantly, signalling via TGF-β (that is derived from the tumour cell) activates normal fibroblasts, driving them to a CAF phenotype (Fig. 8.1). Given that GPC-1 is expressed on fibroblasts found adjacent to pancreatic tumour cells in biopsy tissue [18], we postulate that GPC-1 likely modulates fibroblast activation in PDAC. In support of this idea, stromal cells from GPC-1^{null} Kras-driven pancreatic tumour-bearing mice express less Ki67 (a marker of proliferation) than those from GPC-1-expressing tumour-bearing mice, suggesting GPC-1 controls growth signalling in these cells [21].

In turn, activated CAFs secrete HGF and FGF which drive cancer cell proliferation, migration, invasion and metastasis. Moreover, these signalling molecules can act in an autocrine manner, driving CAF proliferation [63]. Indeed, fibroblasts isolated from GPC-1^{null} mice were unresponsive to stimulation with FGF in a cell migration assay [21]. Glypican-1 may influence growth factor signalling in CAFs, with immediate effects on CAF biology, but also with knock-on effects for tumour cell signalling via inhibition of CAF growth factor release.

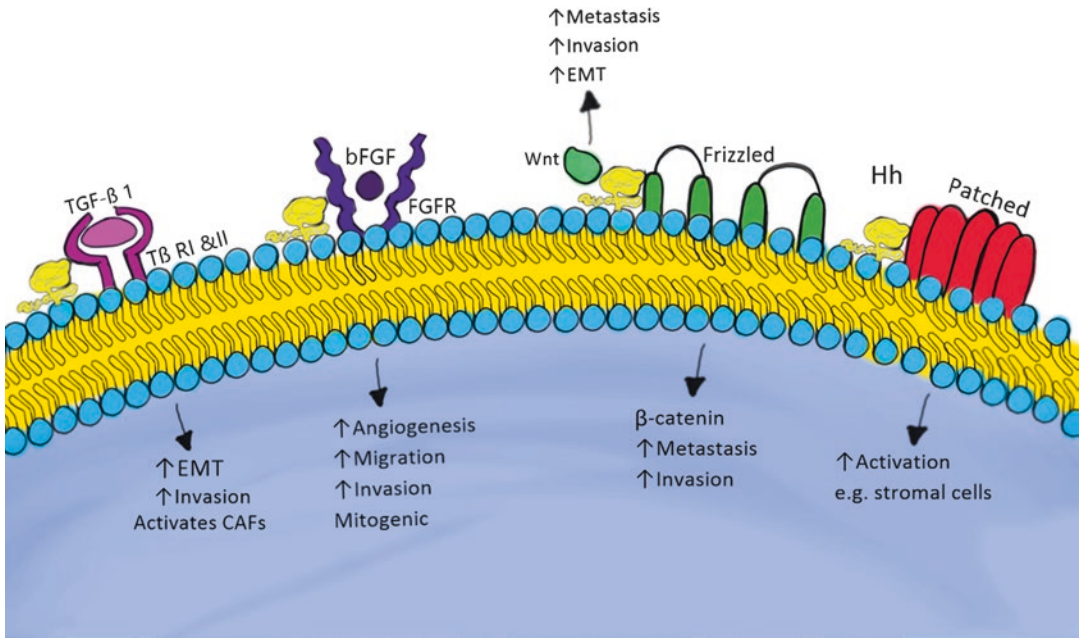

Fig. 8.1 The role of GPC-1 in cell signalling – modulation of factors critical to tumour progression Glypican-1 acts as a co-receptor for TGF-β; signalling via TGFR1 and TGFR11 promotes epithelial-to-mesenchymal transition (EMT) and invasion in tumour cells. TGF-β activates CAFs which influence the tumour and TME. Glypican-1 sequesters FGF in proximity to FGFR driving signalling via the FGF pathway which promotes angiogenesis, drives migration and invasion and is mitogenic in tumour cells. Delivering Wnt to FRIZZLED, GPC-1 influences the β-catenin signalling pathway which drives metastasis and invasion. Hedgehog signalling is influenced by GPC-1 and promotes the activation of TME cells, e.g. stromal cells. TGF-β transforming growth factor-β, EMT epithelial-to-mesenchymal transition, CAFs cancer-associated fibroblasts, Hh Hedgehog, FGF fibroblast growth factor

The Hedgehog (Hh) signalling pathway plays a crucial role in normal development. In pancreatic cancer, it is known to drive the formation of the stroma by activating PSCs [64], and inhibition of Hh signalling reduces the establishment of the stroma and allows exposure of the tumour to chemotherapy [65, 66]. The Hh ligands, derived from the tumour cell, act in a paracrine manner on the PSCs, driving proliferation [67]. Glypican-1 is known to regulate Hh signalling, acting as a co-receptor for Sonic Hedgehog (Shh) and regulating Hh signalling in normal cells, for example, cholangiocytes and neurons [68, 69]. We postulate that GPC-1 may play a role in controlling Hh signalling in PSCs, thus influencing establishment of the stromal compartment in pancreatic cancer (Fig. 8.2).

8.6 Perspective: Glypican-1 as a Therapeutic Target

The critical role of GPC-1 in modulating tumour biology and the TME, as well as its overexpression in a variety of solid tumours and link to poor clinical prognosis, suggests its potential as a

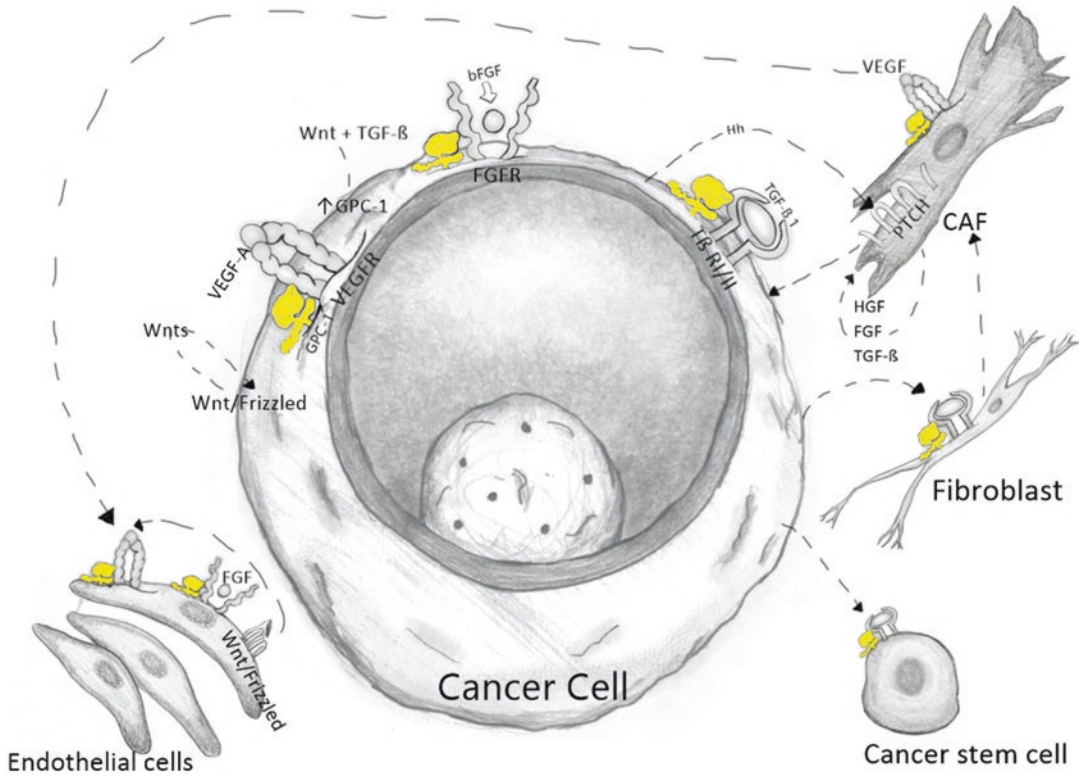

Fig. 8.2 The role of GPC-1 in cell signalling in the tumour and its microenvironment. Glypican-1 is expressed on the surface of endothelial cells, on fibroblasts, on the tumour cell itself and, putatively, on cancer stem cells. Glypican-1 acts as a co-receptor for various signalling molecules, influencing signalling via Wnts, VEGF, FGF and Hedgehogs (Hhs), molecules which may act in autocrine or paracrine manner. In endothelial cells, GPC-1 enhances FGF and VEGF signalling and modulates Wnt signalling which in turn influences VEGF gene expression levels. Fibroblasts differentiate into CAFs in response to tumour cell-derived TGF-β, signalling via which in fibroblasts is controlled by GPC-1. The activation of CAFs leads to the release of HGF, FGF and TGF-β, all of which act in an autocrine manner (regulated by GPC-1 as a co-receptor) or act on tumour cells to drive tumour cell migration, invasion and metastasis. Cancer-associated fibroblast (CAF)-derived VEGF also acts on endothelial cells to drive angiogenesis. Hedgehogs interact with pancreatic stellate cells (PSCs) to drive proliferation. CAF cancer-associated fibroblast, GPC-1 glypican-1, Hh Hedgehog, PSCs pancreatic stellate cells

therapeutic target. Indeed, several studies have demonstrated the efficacy of targeting GPC-1 in different cancers. Knockdown of GPC-1 reduces the proliferation, migration and invasion of breast and pancreatic cancers and inhibits tumour growth in vivo [3, 4, 18]. Targeting of GPC-1 with an antibody inhibits tumour growth through the direct action of the antibody (as opposed to CDC or ADCC). The safety of targeting GPC-1 is evidenced by several studies. Protein expression of GPC-1 in normal adult tissue is limited [1, 2]. Moreover, safety studies in mice using a high dose of anti-GPC-1 antibody (50 mg/kg) that recognises mouse GPC-1 showed no adverse effects associated with targeting of the antigen [2, 15].

Given the crucial role for growth factor signalling pathways in tumour progression, many therapies have been designed to modulate individual signalling pathways. Inhibition of FGFR1 and FGFR2 in pancreatic stellate cells using a silencing RNA or chemical inhibitor has been shown to reduce proliferation and, in organoid cultures, prevents invasion in both pancreatic stellate cells and pancreatic tumour cells [63]. Complete ablation of FGFR in stellate cells completely inhibited invasive ability of the cancer. Inhibition of HGF using a neutralising antibody (AMG102) hindered tumour growth in vivo in an orthotopic model of pancreatic cancer to the same degree as gemcitabine, but had a more profound effect on angiogenesis and metastasis than the chemotherapeutic treatment [70]. Interestingly, this inhibition of metastasis was lost when the HGF inhibitor therapy was combined with gemcitabine. The authors postulated that this was likely due to selection by gemcitabine for a stem-like population more inclined to EMT. Therapeutic targeting of TGF-β signalling using blocking antibodies or anti-sense oligonucleotides (for blocking of ligand and/or receptor) has delivered complex experimental results. In pancreatic cancer, the TGFβR1 small molecule inhibitor galunisertib inhibited proliferation to some degree in cell line studies, but its real effect was in vivo inhibition of invasion [71, 72]. However, the pitfalls of targeting a single pathway therapeutically were highlighted by in vitro studies in 3D tumour-stroma cultures of pancreatic stellate and tumour cells. In fibroblasts, cancer cell-derived TGF-β inhibits HGF secretion; thus, "therapeutic" inhibition of TGF-β signalling led to an increase in fibroblast HGF secretion and, consequently, increased tumour cell invasion, reminding us of the complex interplay between tumour cells and the TME [73].

Despite promising preclinical data, therapies aimed at inhibiting growth factor signalling have had varied clinical success. In pancreatic cancer, inhibition of the Hh pathway was predicted to inhibit PSC growth, thus making the tumour more accessible to gemcitabine. Efficacy was expected based on promising preclinical studies [66]; however, when tested in phase II clinical trials in combination with gemcitabine, the Hh inhibitor saridegib did not show benefit beyond gemcitabine alone (NCT01130142; [74]). Similarly, sulindac, an approved nonsteroidal anti-inflammatory drug (NSAID) known to target the accumulation of β-catenin in the nucleus and influence Wnt signalling, failed to show clinical benefit in lung cancer in phase II clinical trials [75]. These clinical results may not be wholly unexpected, given the complex interplay of tumour and TME and the many signalling pathways involved. Indeed, it is now known that some signalling pathways collaborate to drive tumour progression, which is a difficult phenomenon to target therapeutically using single agents, in part not only because of the complex interplay between pathways but also because our understanding of these complexities is limited. Glypican-1 broadly modulates multiple signalling pathways critical to tumour growth and progression, in both the tumour cells and the TME, whilst not being required for normal homoeostasis, making targeting GPC-1 an attractive therapeutic approach. A blocking antibody or GPC-1 binding peptide may be an appropriate therapeutic agent. Indeed, there is preclinical evidence for the therapeutic potential of blocking glypican family members. Blocking of GPC-3 with the humanised monoclonal antibody HS20 inhibits Wnt signalling and prevents liver tumour growth in vitro and in vivo in nude mice [76].

Targeting of GPC-1 may inhibit fibroblast activation and CAF establishment of the stroma

resulting in a more vulnerable tumour, through modulation of various signalling pathways including FGF and Hh (which may be a superior approach to therapeutic targeting of a single pathway). Moreover, inhibition of CAF activation inhibits the release of TGF-β, critical for maintaining immune suppression in the tumour, as well as driving tumour cell EMT. TGF-β signalling in CAFs is thought to promote the production of extracellular matrix (ECM), establishing fibrosis. Indeed, TGFβR1 inhibition with kinase inhibitor SD-208 in an orthotopic PANC-1 xenograft reduced tumour growth and fibrosis [77]. Moreover, Hh signalling, another pathway mediated by GPC-1, drives fibrosis [64]. Thus, targeting of GPC-1 may be a means to inhibit fibrosis. Reduction in CAF-released FGF and HGF inhibits tumour cell proliferation, migration, invasion and metastasis and autocrine CAF activation. Inhibition of GPC-1 signalling (FGF, VEGF and Wnt) inhibits angiogenesis, crucial to tumour survival, by modulating the function of endothelial cells. Finally, targeting of GPC-1 signalling inhibits tumour cell growth factor signalling required for growth, invasion and metastasis, including signalling via FGF, HGF, TGF-β and Wnt, and may modify the response to collaborative pathways such as Wnt/TGF-β. The broad inhibition achieved by blocking of GPC-1 would likely inhibit tumour growth, invasion and metastasis, in line with experimental work demonstrating the therapeutic effects of GPC-1 knockdown or blocking in vitro and in mouse studies and clinical evidence demonstrating a role for GPC-1 in tumour invasion. Work performed by our collaborators has demonstrated that knockdown of GPC-1 by siRNA inhibits proliferation, migration and invasion of a variety of prostate cancer cell lines in vitro (manuscript in preparation). Importantly, in vivo, knockdown not only inhibits the growth of subcutaneous prostate cancer tumours but also inhibits metastasis in a PC3 intracardiac metastatic model (manuscript in preparation).

8.7 GPC-1 and Cancer Stem Cells

Cancer stem cells are a subpopulation of cancer cells that are critical to tumour recurrence and to the establishment of metastases. Signalling via TGF-β modulates the function of CSCs, including driving EMT. For example, in glioblastoma, the stem-like population glioma-initiating cells (GICs) rely on TGF-β for self-renewal, through activation of the JAK-STAT pathway [78]. Thus, targeting of GPC-1 may inhibit the function of CSCs via modulation of TGF-β signalling. For some indications, the CSC population plays a more prominent role in pathogenesis, for example, in triple-negative breast cancer (TNBC). This phenotype of breast cancer has a worse prognosis than the ER- and HER2-positive tumour phenotypes, and this is thought to be related to the presence of virulent CSCs [79]. There is precedence for targeting of the TGF-β pathway in this indication, as in vitro work using genetic or pharmacological inhibitors of TGF-β prevented the expansion of CSCs [80]. The expression of GPC-1 in triple-negative breast cancer has not yet been described; however, this is a subject of investigation in our lab. If expression is high, then a GPC-1 blocking strategy may be therapeutically appropriate for this indication.

Glypican-1 blocking therapies may have potential in combination with standard-of-care therapies. Anti-oestrogens (e.g. tamoxifen) are used as an adjuvant treatment for oestrogen receptor-positive (ER^+) breast cancers; however, resistance is commonplace, developing in one-third of patients and close to all patients with metastatic disease [81]. It has been postulated that signalling via FGF promotes resistance to anti-oestrogen therapy, through upregulation of cyclin D and downregulation of the pro-apoptotic factor Bim [82]. Blockade of FGFR or FGFs reversed drug resistance in murine PDX models of drug-resistant breast cancer. Given the critical role of GPC-1 in promoting FGF signalling, there is a clear rationale for a combination of a GPC-1 targeting therapeutic with hormone therapy in hormone-responsive breast cancers [83].

In summary, GPC-1 plays a critical role in the tumour and TME, modulating tumour growth

factor signalling, influencing angiogenesis and controlling activity of components of the stroma, including CAFs. The end point of these complex interactions is the promotion of tumour growth, invasion and metastasis. Importantly, in adults, modulation by GPC-1 is largely restricted to tumour sites, so therapeutic targeting of GPC-1 would not affect normal homoeostasis (an idea supported by preclinical safety studies). Thus, targeting of GPC-1 may provide an effective, broad-ranging therapeutic strategy to address multiple underlying issues in the tumour and TME, including oncogenic growth factor signalling, establishment of angiogenesis and therapy-resistant CSCs that contribute to disease recurrence.

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