Biology of Extracellular Matrix 11
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The Extracellular Matrix and the Tumor Microenvironment

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Biology of Extracellular Matrix

Volume 11

Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

All chapters are systematically reviewed by the series editor and respective volume editor(s).

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The Extracellular Matrix and the Tumor Microenvironment

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Preface

The types of malignant tumors are determined by the tissue the transformed cells originate from. However, without the establishment of tumor stroma, tumor tissue could not exist. Accumulating data of cancer research shed light to the importance of the stromal components supporting the growth and survival of tumor cells. In the last 20 years, huge amounts of experimental and clinical data have been collected about the subject. Although dysregulated, cancer is an ecosystem, where cancer cells coexist with a plethora of non-tumorous cells, and blood vessels, embedded into the extracellular matrix. All of these components are in intensive communication with each other via membrane receptors and soluble factors. Furthermore, matrix proteins also have regulatory potential. Thus, to understand their interplay with the tumor cells is mandatory to step forward.

Extracellular matrix is an acellular substance synthesized by its resident cells. Its two major forms are the basement membrane and the interstitial matrix built up by structural proteins such as collagens glued together with adhesive glycoproteins, laminins, and fibronectins. Former is a sheet-like structure that provides support for various epithelial layers, or surrounds individual stromal cell like chondrocytes. Interstitial matrix is a lattice-like formation built up by collagens and fibronectins. It provides support for the resident stromal cells, blood vessels, and the epithelial cell of various organs. Proteoglycans can be found in the basement membranes, interstitial matrix, and the surface of epithelial cells. They are active members of the events taking place in the ECM. Owing to their negatively charged sugar chains, they can interact with regulatory factors, or even exist as cell surface receptors. Hyaluronic acid is responsible for the maintenance of tissue turgor. However, these molecules and several other residents of the extracellular matrix are capable to join to the wrong side and support the development and progression of cancer.

This book was created with the intention to provide examples as the results of the last 20 years altered our understanding about the role of the extracellular matrix in health and disease, including cancer. Now we know that ECM is not an inert material, but active participant of the events taking place in our body. These

advancements will be presented in eighteen chapters from scientists around the world.

The **first five chapters** are dealing with proteoglycans, built up by a protein core, to which negatively charged glycosaminoglycan sugar chain(s) are covalently attached. Their effect can be beneficial, or harmful, depending on the nature of their components. Furthermore, **Chap. 5** provides an excellent example about versikine, a tryptic fragment of versican. Protease activity can liberate fragments of proteoglycans, which entering into the circulation can be utilized in diagnosis and may be in therapy as well, as it is demonstrated by **Chap. 6.** As it is presented in **Chap. 7,** hyaluronan can promote the progression of hematological and solid solid tumors. Heparanase is the sole endoglycosidase, degrading heparan sulfate chains of proteoglycans. Its activity, that can be detected in almost all types of cancer, is an indicator of poor tumor prognosis is discussed in **Chap. 8**.

The next two **chapters (Chaps. 9 and 10)** provide information as laminins, fibronectins, and collagens support tumor progression. **Chapter 11** demonstrates how matrix receptor integrins transmit information from the matrix to the tumor cells and cancer associated fibroblasts (CAFs). Unexpected negative effect of fat on the behavior of mammary cancer is reported in **Chap. 12**.

Chapter 13 sheds light on the cooperation of ECM and tumor cells in the impaired intermediary metabolism. Tumor tissue dies without blood supply. Mechanisms of cancer angiogenesis and its influencing factors are introduced in **Chaps. 14 and 15.**

Epigenetic regulation is also involved in the cooperation of extracellular matrix and tumor cells as it is demonstrated in **Chap. 16** in case of breast cancer.

Finally, the last two **chapters (Chaps. 17 and 18)** try to convince the readers, that targeting components of extracellular matrix may have supporting potential in the battle against cancer in the future.

Budapest, Hungary — Ilona Kovalszky
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The editors would like to thank to the contributors of the book, who are well-known experts of the fields. The fruits of their research, they shared with us, opens up the vivid word of extracellular matrix and stromal components indispensible for health and disease.

We are grateful for the stimulating suggestions and problem-solving interventions of our Series Editor professor Nikos Karamanos and the Associate Editor Dr Miriam Latuske, and, last but not least, for everybody from the side of Springer-Nature who helped us in the whole process to turn our chapters into a book.

Contents

Chapter 1
Small Leucine-Rich Proteoglycans Regulate Cancer Cell Growth, Apoptosis, and Associated Inflammation

Dragana Nikitovic and George Tzanakakis

Abstract The tumor microenvironment, apart from tumor cells, contains tissue nonmalignant cells, blood vessels, stromal cells, infiltrating immune cells, and the modified extracellular matrix (ECM). The altered ECM essentially regulates all tumor cells' functions and forms a permissive tumor cell environment. Proteoglycans (PGs) are an essential component of the ECM and correspond to composite molecules that comprise a protein core into which one or more glycosaminoglycans (GAG) chains are covalently bound. Small leucine-rich proteoglycans (SLRPs) are a family of 18 proteins. The SLRPs are grouped into five classes based on their homology at both the protein and genomic levels, the number of the leucine-rich repeats (LRRs), the spacing of their N-terminal cysteine residue and ear repeats, and crucially their shared functional features taking into account that some SLRPs are not classical proteoglycans. Due to their structure's specificities, the SLRPs have been indicated to participate in myriad physiological and pathological processes. This chapter provides a critical overview of biglycan and lumican roles in the reactive cancer milieu and consecutively extrapolates to potential options for developing targeted treatments.

1.1 Introduction on Cancer and Its Microenvironment

The process of carcinogenesis is highly complex with the participation of genetic, metabolic, and environmental factors (Tzanakakis et al. 2019, 2020). The malignant transformation of the cancer cells bestows them with more efficient growth, survival, motility, metastasis, and the ability to reprogram their microenvironment. Further expansion of the tumor cells is facilitated through their constant interactions with the altered tumor microenvironment (TME) (Nikitovic et al. 2015). The TME, apart from tumor cells, contains tissue nonmalignant cells, blood vessels, stromal cells,

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infiltrating immune cells, and the modified extracellular matrix (ECM) (Joshi et al. 2021; Suveges et al. 2020). The altered ECM essentially contributes to all tumor cells' functions and forms a permissive tumor cells environment (Nikitovic et al. 2018; Pickup et al. 2014). The ECM supramolecular structure is composed of fibrillar proteins such as collagen and elastin, proteoglycans (PGs), and GAGs, with the ability to specifically support the structure/function of each organ (Hynes 2009).

Thus, the altered ECM supports all the "hallmarks" of cancer as defined by Hanahan and Weinberg (2000), including sustained proliferation, evasion of growth suppression, death resistance, replicative immortality, induced angiogenesis, initiation of invasion, dysregulation of cellular energetics, avoidance of immune destruction, and chronic inflammation (Hanahan and Weinberg 2000). Indeed, the tumor ECM contributes to the tumor development and sustains the tumor immune escape forming an immunosuppressive scaffold where stromal cells intertwine with inflammatory immune cells and cells responding to the vascular system (Dunn et al. 2004). In addition, tumors commonly exhibit desmoplasia, an increased deposition and cross binding of the ECM proteins (Lu et al. 2012). Likewise, fibrosis characterized by abnormal accumulation of collagen (Sorensen et al. 1998) and increased mammographic density due to the enhanced collagen deposition (Morris et al. 2016) are correlated to cancer progression. Thus, the "stiffening" of the ECM due to the alteration of its components enhances tumor cell functions.

PGs are an essential component of the ECM. These composite molecules comprise a protein core into which one or more GAG chains are covalently bound. The attached GAGs types differ as heparan sulfate (HS), chondroitin sulfate/dermatan sulfate (CS/DS), or keratan sulfate (KS) chains can decorate the PG protein core. The localization of PGs varies as they can be associated with the plasma membranes, secreted into the ECM, or deposited inside the cells. Secreted PGs are localized to the pericellular space and the "proper" ECM where the PGs are localized away from cells. Forty-five PGs have been identified up to now, endowed with huge variability in the protein core and the glycosylation pattern (Iozzo and Schaefer 2015).

PGs expression pattern and regulatory roles are altered during tumorigenesis and have been closely correlated to cancer development and progression as previously discussed (Tzanakakis et al. 2019; Nikitovic et al. 2018). It is well established that malignant tumors have discrete PG expression profiles immediately correlated with their behavior and differentiation status. Thus, epithelial tumors exhibit a different PG phenotype compared to mesenchymal tumors (Mytilinaiou et al. 2017). The proteolysis of PGs, performed by matrix metalloproteinases (MMPs), cathepsins, and bone-morphogenetic protein-1, can liberate bioactive matrikines with different roles in tumorigenesis compared to original molecules (Pasco et al. 2004; Hope et al. 2017). Moreover, PGs are closely involved in cancer-associated inflammation (Tzanakakis et al. 2021).

1.2 SLRPS Structure and Function-Focus on Biglycan and Lumican

Small leucine-rich proteoglycans are a family of 18 proteins. Their protein core, characterized by a variable number of central leucine-rich repeat (LRR) domains, has a molecular weight between 36 kDa and 77 kDa and a horseshoe shape. LRRs exhibit different amino acid sequences across the SLRP family, their size being in the 20 to 29 residues range, whereas the N and C-terminal regions of the protein have abundant cysteine residues (Iozzo and Schaefer 2015; McEwan et al. 2006; Kobe and Kajava 2001). The penultimate of the two C-terminal LRRs is the ear-repeat characteristically being the longest LRR and protruding laterally from the core protein's main axis (Iozzo and Schaefer 2015; McEwan et al. 2006). The specific residue sequences of the LRRs, the glycosylation pattern, the number of cysteine, and the ear-repeats regulate protein structure and are involved in ligand recognition. The concave SLRPs face is formed of β-sheets, while α-helices contribute to the formation of the convex surface. Moreover, each LRR supplies one α-helix and one β-sheet that correlate to a one-turn tube-like structure, with the LRR hydrophobic residues facing inside (Scott et al. 2004). The resulting structure facilitates protein–protein interactions with the inner concave face and enables the detainment of a ligand.

Furthermore, the majority of SLRP family members undergo different post-translational glycosylation. Since SLRPs core proteins bear a multitude of substitution sites corresponding to a variable glycosylation status, this provides an exquisite specificity of SLRP interactions with diverse cell membrane receptors, cytokines, chemokines, and ECM molecules (Kram et al. 2017).

The 18 SLRP members are grouped into five classes based on their homology at both the protein and genomic levels, the number of the LRRs, the spacing of their N-terminal cysteine residue and ear repeats, and crucially their shared functional features taking into account that some SLRPs are not classical proteoglycans (Schaefer and Iozzo 2008; Dellett et al. 2012).

Indeed, the canonical class I members, decorin and biglycan, bear CS/DS side chains, whereas the asporin member does not (McEwan et al. 2006; Henry et al. 2001). In contrast, the class II members, such as lumican, are decorated by KS chains or polylactosamine bound into LRRs, while class III members can bear KS (osteoglycin), CS/DS (epiphycan), or be bereft of GAG (opticin) chains (Schaefer and Iozzo 2008; Sanders et al. 2003). Unexpectedly, the majority of the noncanonical classes IV and V members (Iozzo and Schaefer 2015) do not carry GAG chains, excluding chondroadherin which is modified with KS (Neame et al. 1994). In continuation, we will focus on the class I member biglycan and the class II member lumican structure biological roles in the tumor microenvironment (TME).

1.2.1 Biglycan and Lumican Structure

The biglycan 42-kDa protein core contains 10 to 12 leucine-rich repeats (LRRs), flanked by cysteine-rich regions. Human biglycan carries 1 to 2 GAG chains bound into sites at amino acids 5 and 10 of the N-terminus (Roughley and White 1989). The substitution is implemented by CS or DS chains in a tissue-specific manner (Roughley and White 1989). Moreover, biglycan can be expressed as a "part-time" PG considering its nonglycated form has been identified in the aging articular cartilage. The nonglycanated biglycan forms seem to result from the proteolysis of the core protein's amino-terminal region (Roughley et al. 1993). Moreover, biglycan possesses N-linked oligosaccharide chains within its central LRRs (Neame et al. 1989). The gene for biglycan is located at the Xq28 (McBride et al. 1990). The details of biglycans' structural characterizations have previously been excellently discussed (Schaefer and Iozzo 2008).

Biglycan has a significant role in the organization of ECM assembly as it interacts explicitly with various ECM components, including collagen types I, II, III, IV, and VI and elastin (Douglas et al. 2006), thereby playing a crucial structural role in the majority of tissues (Wiberg et al. 2003; Reinboth et al. 2002; Kram et al. 2020).

Lumican, a class II SLRP, has a 40-kDa core protein that bears 6 to 10 LRR. This SLRP was initially determined to be a significant KS-containing PGs of the chick cornea (Blochberger et al. 1992; Schrecengost et al. 1992). Moreover, lumican can also be substituted by polylactosamine chains of varying sizes or be bereft of glycosylation (Melching and Roughley 1999). Furthermore, it has been suggested that nonglycanated forms of lumican tend to increase with age due to the attenuation of KS synthesis (Roughley et al. 1996).

Notably, the function of the SLRPs depends on both their core protein and GAG chains (Nikitovic et al. 2008a, 2008b, 2012). Indeed, the SLRPs core proteins interact with the fibrillar collagen, mainly responsible for the tissue scaffold formation (Iozzo and Schaefer 2015). By binding, the SLRPs regulate the fibril diameter formation and their interactions with other ECM components (Svensson et al. 2000). Moreover, the targeted knock-down of the BGN gene leads to irregular morphology of the collagen fibril (Ameye et al. 2002). They also appear to control access of the collagenases to their unique cleavage site and thus, partly modulate collagen proteolysis (Monfort et al. 2006).

This chapter provides a critical overview of biglycan and lumican roles in the reactive cancer milieu and consecutively extrapolates to potential options for the development of targeted treatments.

1.3 Lumican and Biglycan Expression in Cancer and Correlation to Carcinogenesis

Biglycan and lumican exhibit altered expression in cancer tissue. Thus, biglycan is overexpressed in endometrial cancer tissue samples, both at the parenchyma and mesenchyme, compared to human normal and atypical hyperplasia endometrium (Liu et al. 2014). Furthermore, the expression of biglycan in cancerous endometrium mesenchyme was positively correlated with poor patient prognosis (Liu et al. 2014). Likewise, biglycan was found to be upregulated at both the protein and mRNA level in gastric cancer tissue compared to adjacent nontumorous tissues. Moreover, patients with biglycan-positive tumors exhibited higher disease recurrence rates and increased mortality than patients with biglycan-negative tumors following surgery (Wang et al. 2011). Indeed, it seems that biglycan mRNA is overexpressed in the majority of cancer tissues compared with normal tissues, including breast, bladder, brain and central nervous system, gastric, colorectal, esophageal, lung, head and neck, ovarian, and 28 subtypes of cancer determined by a recent study examining the Oncomine database (Zhao et al. 2020).

Notably, biglycan was a significant differentially upregulated gene correlated to gastric cancer metastases (Liu et al. 2021). Furthermore, Wei et al. (2020), utilizing the GSE75037 published data, had built the coexpression modules of genes by Weighted Gene Co-Expression Network Analysis (WGCNA) to evaluate the function and protein–protein interaction network of coexpression genes by Database for Annotation, Visualization, and Integrated Discovery (DAVID) and String database, respectively, in lung cancer. This approach determined that biglycan is one of the critical genes facilitating the occurrence of lung adenocarcinoma through putative ECM–receptor interaction (Wei et al. 2020).

In general, a good correlation was established between biglycan mRNA and protein expression in various cancer tissues, including bladder (Schulz et al. 2019; Appunni et al. 2017), esophagus (Zhu et al. 2013), or colorectal cancer (Gu et al. 2012). Thus, Zhao et al. (2020) data agree with the previous report showing that biglycan protein overexpression is correlated to poor patient prognosis in esophagic cancer (Zhu et al. 2013) and bladder cancer (Schulz et al. 2019). Biglycan was found to be overexpressed in approximately a third of prostate cancer tissues and Jacobsen et al. (2017) show that biglycan expression parallels disease progression (Jacobsen et al. 2017).

Some studies, however, had contrasting results. Indeed, in a cohort of 76 bladder cancer patients, high biglycan expression was correlated to reduced tumor cell proliferation and increased 10-year survival of patients (Niedworok et al. 2013).

Immunohistochemistry demonstrated a much higher expression of lumican in gastric cancer tissues compared to adjacent normal gastric. Moreover, lumican expression was correlated with histological type, organ metastasis, and lymphatic metastasis (Chen et al. 2017). Furthermore, upon downloading the gene expression profiles and gastric cancer patients' clinical data from The Cancer Genome Atlas (TCGA) database, a significant difference in lumican expression was identified

between gastric cancer tissues and adjacent nontumor tissues by R software and quantitative real-time polymerase chain reaction (qRT-PCR) as well as subsequent comprehensive meta-analysis (Chen et al. 2020a, 2020b). Moreover, when multivariate analysis was applied, high lumican expression was identified as an independent predictor of poor overall survival (Chen et al. 2020a, 2020b).

In breast cancer, lumican mRNA was identified in the tumor stroma and correlated with higher tumor grade, lower expression of estrogen receptors, and younger age of the patients (Leygue et al. 1998). Lumican expressed in breast cancer cell lines affected their biological behavior and expression of ECM mediators (Karamanou et al. 2020). In colon cancer, lumican expression was correlated with lymph node metastasis and the extent of tumor invasion (Seya et al. 2006). Moreover, patients with a high lumican expression exhibited a lower survival rate (Seya et al. 2006).

Lumican was likewise detected in malignant melanoma's dermis and peritumoral stroma but not identified in melanoma cells (Brezillon et al. 2007). Indeed, lumican was suggested to negatively regulate the vertical progression of melanoma (Brezillon et al. 2007). Moreover, lumican was found to inhibit the metastasis of melanoma in vivo by modifying matrix effectors and invadopodia activity (Karamanou et al. 2021). On the other hand, some human melanoma cell lines were found to express lumican mRNA and secrete lumican, which could be attributed to cell culture conditions (Sifaki et al. 2006).

1.3.1 Biglycan and Lumican Affect Tumor Growth

Numerous studies have established a correlation between biglycan and lumican expression and cancer cell growth. Both positive and negative effects, putatively in a cancer-type dependent manner, have been identified. Thus, HCT116 colon cancer cells deficient in biglycan exhibit downregulated cell growth and cell cycle arrest at the G0/G1 phase. This was followed by a decrease in positive cell cycle regulators, including cyclin A and cyclin D1, and increased expression of negative cell cycle regulators such as p21 and p27 (Xing et al. 2015a, 2015b). Moreover, biglycan was shown to upregulate the growth of MG63 osteosarcoma cells through an LRP6/β-catenin/IGF-IR signaling axis with the participation of Erk1/ downstream signaling (Aggelidakis et al. 2018, 10.3389/fonc.2018.00470).

On the other hand, human urothelial carcinoma cells (J82 cells) deficient in biglycan exhibit enhanced growth, whereas biglycan overexpression and treatment with exogenous biglycan attenuated these cell proliferation. Likewise, a xenograft of biglycan-deficient J82 cells exhibited increased growth in nude mice (Niedworok et al. 2013). Moreover, Kaplan–Meier analysis indicated higher 10-year survival of patients whose biopsies showed high biglycan mRNA expression (Niedworok et al. 2013).

Notably, biglycan was one of the PG components of the desmoplastic reaction. Furthermore, it was shown that biglycan, highly expressed in pancreatic cancer, attenuates this cancer cells' growth by inducing G1-arrest (Weber et al. 2001).

The majority of studies report inhibiting effects of lumican on cancer growth. Thus, lumican was shown to prolong the doubling time and inhibit the cell growth in H460 and A549 non-small lung cancer cells. Moreover, lumican was demonstrated to inhibit the central spindle and midbody formation during cytokinesis leading to increased aneuploidy. Indeed, Yang et al. (2020) suggest that lumican controls chromosome segregation during cell division in the in vitro lung cancer model (Yang et al. 2020).

Activated pancreatic stellate cells (PSC) are the dominant stromal cell type in the microenvironment pancreatic ductal adenocarcinoma (PDA), primarily responsible for the deposition of collagen (Apte et al. 2004). Lumican secreted by PSCs cells to the PDAC ECM (Kang et al. 2016) is associated with improved patient outcome after surgical removal of localized PDAC and multimodal therapy (Li et al. 2014). Furthermore, in pancreatic ductal adenocarcinoma cells, extracellular lumican attenuated the EGFR expression and phosphorylation by inducing dimerization and internalization of EGFR, leading to the downregulation of Akt kinase activity and reduced growth. This model mimics the interaction between cancer and stromal cells resulting in the limiting of cancer growth (Li et al. 2014).

In a separate study, the prolonged exposure of pancreatic ductal adenocarcinoma cells to exogenous lumican initiated growth inhibition and G0/G1 arrest, which characterize cancer cell quiescent state. These effects were retained in xenograft and syngeneic orthotopic animal models, where lumican attenuated pancreatic tumor growth (Li et al. 2017). Moreover, a sequence of 17 amino acids within the lumican core protein, named lumcorin, strongly attenuated the monolayer and soft agar growth of mice B16F1 and human SK-MEL-28 melanoma cells (Pietraszek et al. 2013).

Interestingly, the growth of human osteosarcoma Saos 2 cells, characterized as nonaggressive, was downregulated by lumican, whereas the proliferation of the aggressive MG63 human osteosarcoma cells was not affected (Nikitovic et al. 2008a, b). The expression of lumican expression was negatively correlated with the Smad2 basal level in these cells, indicating that this SLRP may affect the bioavailability of Smad 2 activators (Nikitovic et al. 2008a, b).

On the other hand, some growth-promoting effects of lumican have been identified. An example is gastric cancer, where it was determined that lumican produced by cancer-associated fibroblasts promotes this cancer cell growth through integrin β1-FAK signaling pathways. Knock out of lumican attenuated gastric tumor growth and metastasis in vivo (Wang et al. 2017). Moreover, lumican was shown to upregulate the growth of HTB94 chondrosarcoma cells through an IGF-IR/Erk1/2 axis (Papoutsidakis et al. 2020). Some effects of biglycan and lumican on cancer cell growth are schematically depicted in Fig. 1.1.

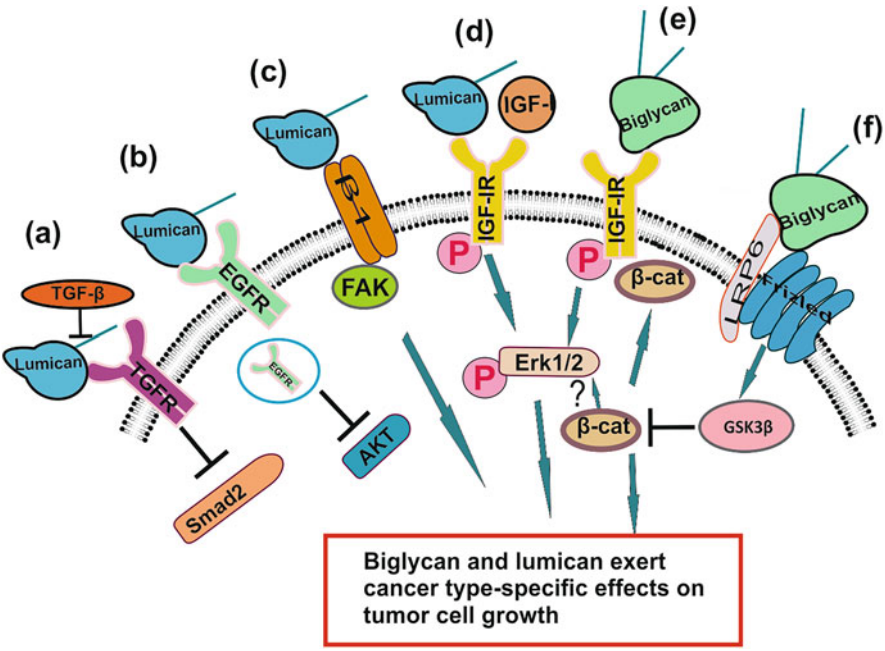

Fig. 1.1 Effects of biglycan and lumican on cancer cell growth. (**a**) Lumican inhibits osteosarcoma cell growth by affecting Smad2 signaling. (**b**) In pancreatic cancer, lumican attenuates the EGFR expression and phosphorylation by inducing dimerization and internalization of EGFR, leading to the downregulation of Akt kinase activity and reduced growth. (**c**) In gastric cancer, lumican produced by cancer-associated fibroblasts promotes this cancer cell growth through integrin β1-FAK signaling pathways. (**d**) Lumican stimulates the growth of HTB94 chondrosarcoma cells through an IGF-IR/Erk1/2 axis. (**e**) Biglycan enhances the growth of MG63 osteosarcoma cells through an LRP6/β-catenin/IGF-IR signaling axis with the participation of Erk1/ downstream signaling

1.3.2 Effects of Biglycan and Lumican on Cancer Cell Motility and Invasion

While cell migration is a normal process necessary in embryogenesis, tissue homeostasis, and regeneration, cell invasion is perpetrated during the penetration of biological barriers and metastasis (Condeelis and Segall 2003). Cells can migrate utilizing an amoeboid motion exerting weak adhesion and traction. Alternatively, cells migrate through a mesenchymal movement by forming cytoplasmic protrusions, adhering to the ECM and performing cell body translocation. The mesenchymal mode also includes extracellular proteolysis with the widening of pores and gaps (Condeelis and Segall 2003; Friedl and Wolf 2010). Cell 2D migration is facilitated by forming various locomotory cytoplasmic protrusions including filopodia, pseudopodia, and lamellipodia, promoting the "gliding" of the cells on the substrate (Taylor and Condeelis 1979). On the other hand, in 3D movement, cells form

invasive plasma membrane protrusions such as invadopodia, able to adhere in an integrin-dependent manner on the ECM but also to degrade the ECM by secreting matrix metal proteinases (MMPs) (Génot and Gligorijevic 2014; Di Martino et al. 2016).

Notably, the creation of both invadopodia and pseudopodia in the tumor microenvironment is controlled by stromal cells such as macrophages and fibroblasts (Parekh et al. 2011; Gaggioli et al. 2007).

Lumican has been shown to inhibit invadopodia and lamellipodia development by prostate cells (Coulson-Thomas et al. 2013). Indeed, when prostate cancer cells were seeded on a lumican substrate, the lamellipodia decrease was detected by an attenuated rearrangement in ZO-1, keratin 8/18, integrin β1, and MT1-MMP, whereas invadopodia attenuation was identified by disruption of α-smooth muscle actin, cortactin, and N-WASP (Coulson-Thomas et al. 2013). Likewise, the inhibition of melanoma cell invadopodia formation was detected in the presence of lumican (Radwanska et al. 2008). Furthermore, lumican abrogates the Snail-dependent MMP-14 activity in B16F1 melanoma cells but not in HT-29 colon adenocarcinoma cells (Stasiak et al. 2016). Moreover, lumican inhibits melanoma cell migration by directly binding α2β1 integrin with its core protein (Zeltz et al. 2010). Furthermore, lumican affects the expression of matrix effectors, inhibits the formation of MMP-releasing invadopodia to inhibit melanoma lung metastasis in vivo, as well as cell invasion in vitro (Karamanou et al. 2021). Lumican downregulates Snail activity, considering that Snail is a significant trigger of epithelial–mesenchymal transition (Moody et al. 2005); this lumican activity could form the base of anticancer therapy.

In a breast cancer model, lumican regulated cell morphology, evoked EMT/MET reprogramming, and inhibited the expression of significant matrix effectors such as MMPs. Moreover, lumican downregulated crucial cell properties, including migration and invasion (Karamanou et al. 2017).

On the other hand, the downregulation of lumican attenuated bladder cancer cells' migration by inhibiting MAPK signaling (Mao et al. 2019). Likewise, the decrease of lumican inhibited the adhesion of lung osteotropic cancer cells to various ECM components, attenuated in vitro cell migration and invasion, and interfered in vivo lung metastasis (Hsiao et al. 2020). Furthermore, the overexpression of lumican by human colon cancer cells enhanced the formation of podosome-like structures and these cells' migration (Radwanska et al. 2012). Lumican facilitated the Saos 2 cell migration and chemotactic response to fibronectin (Nikitovic et al. 2008a, b) and promoted the TGFβ2-dependent adhesion of Saos 2 cells to the fibronectin substrate (Nikitovic et al. 2011).

Desmoplasia, a response of the cancer stroma, is characterized by the unorganized production of fibrous tissue, mainly consisting of collagen fibers and fibroblasts (Kunz-Schughart and Knuechel 2002). The fibrotic tissue is produced chiefly by the "activated" cancer-associated fibroblasts (Kunz-Schughart and Knuechel 2002; Apte et al. 2004). The secretion of the altered ECM can significantly modulate the growth, migration, and spread of cancer cells (Kunz-Schughart and Knuechel 2002). Lumican has been identified to the adjacent stromal tissues in human

pancreatic cancer, and patients with lumican-positive stromal tissues had shorter survival than those with lumican-negative stromal tissues (Ishiwata et al. 2007).

Therefore, as previously summarized, lumican exhibits cancer-specific roles in carcinogenesis, and further in-depth study of its effects is necessary.

The class I SLRP biglycan was likewise shown to affect tumor cell migration and invasion abilities. Notably, biglycan was shown to affect the desmoplastic reaction in colorectal cancer, inhibiting the migration and invasion of colorectal tumor cells in 2D and 3D coculture systems (Coulson-Thomas et al. 2011).

On the other hand, biglycan facilitates endometrial cancer cell invasion and migration in vitro and a xenograft model (Sun et al. 2016). In gastric cancer in vitro and in vivo models, biglycan was demonstrated to enhance tumor invasion and metastasis by facilitating FAK phosphorylation at Tyr576/577, Tyr925, and Tyr397 as of paxillin (Hu et al. 2014). Biglycan also affects mesenchymal origin tumors motility and invasion. Indeed a cooperative mechanism of PTH (1–34) and FGF-2 action leading to an increase in biglycan expression was demonstrated to facilitate osteosarcoma cell migration (Datsis et al. 2011).

Biglycan can exhibit adverse effects on cancer cell motility. Thus, the RAC1B–SMAD3–biglycan signaling axis was found to attenuate the migration of metastatic pancreatic cancer cells (Otterbein et al. 2019). Moreover, biglycan decreases the malignant properties of HER-2/neu-transformed cells, which is inversely correlated to the PKC signaling (Recktenwald et al. 2012).

1.3.3 Biglycan and Lumican at the Crossroad between Apoptosis, Autophagy, and Cancer-Associated Inflammation

The process of tumorigenesis is intimately correlated with chronic inflammation, with a significant 20% of cancer incidences directly related to chronic infections (Aggarwal et al. 2009). All tumor types separately of etiology specifically interact with the immune system at all stages of carcinogenesis (Grivennikov et al. 2010). The ECM components play a significant role in these interactions. Indeed, the tumor microenvironment's extensive remodeling modulates the immune response (Avgustinova et al. 2016). Biglycan, either secreted or proteolytically released, is an established pro-inflammatory molecule and is characterized as a danger signal (DAMP) (Schaefer et al. 2017). Thus, soluble biglycan binds to the Toll-like receptors (TLR)-2 and − 4 on the surface of macrophages, inducing sterile inflammation or enhancing pathogen-mediated inflammation through the induced production of pro-inflammatory mediators (Schaefer et al. 2005). Utilizing TLR2/4 signaling pathways biglycan induces the adaptor molecule myeloid differentiation primary response 88 (MyD88) for the recruitment of neutrophils and macrophages or initiates Toll/interleukin (IL)-1R domain-containing adaptor inducing interferon (IFN)-β (TRIF) activities for T-lymphocyte recruitment (Zeng-Brouwers et al.

2014). Indeed, the binding of biglycan to macrophage CD14, an established GPI-anchored TLRs coreceptor, can induce a discrete response dependent on the TLR type (Roedig et al. 2019).

Notably, biglycan can regulate the generation of radical oxygen species (ROS), which are significant mediators of the tumorigenesis process (Nastase et al. 2017). Upon binding to the TLRs, biglycan was shown to stabilize NADPH oxidase (NOX) 1, 2, and 4 enzymes, modulating their ROS production and subsequently ROS-dependent IL-1β synthesis (Hsieh et al. 2016). Furthermore, the ROS production initiated by biglycan increases the secretion of chemokine (C-X-C) ligand 13 (CXCL13) which acts as an essential chemoattractant for B and B1 lymphocytes (Shaefer et al. 2005). Moreover, biglycan through TLR2/4-dependent cytokine release can induce macrophage and T cells recruitment.

These actions can be both pro- and antitumorigenic. Indeed, biglycan-dependent NOX-generated ROS can cause genomic instability and alterations of chromosomal DNA, which results in enhanced tumor cell growth and metastasis (Wu et al. 2014). On the other hand, biglycan exhibits anti-inflammatory effects promoting the resolution of acute inflammation and the initiation of acquired immunity response (Hsieh et al. 2016). Schaefer et al. (2017) summarize that biglycan actions can modulate both innate and adaptive immunity acting at different checkpoints of the tumor–immune system interactions (Schaefer et al. 2017).

The data on the effects of lumican on tumor-associated inflammation are somewhat limited (Nikitovic et al. 2014). Thus, lumican can affect peripheral monocyte extravasation and Fas–FasL signaling (Wu et al. 2007; Nikitovic et al. 2015).

1.3.3.1 Effects on Apoptosis and Autophagy

Notably, these SLRPs have been indicated in the regulation of autophagy and apoptosis. Indeed, increased expression of biglycan in gastric cancer protects against apoptosis, facilitates cell migration and invasion of tumor cells, and promotes cancer-associated angiogenesis (Pinto et al. 2021). Indeed, it was shown that lumican-deficient gastric cancer cells express increased PARP1, caspase-3 cleavage levels, and decreased mesenchymal markers (Pinto et al. 2021). Supplementation of biglycan-deficient cells with exogenous biglycan restored their survival. In another study, however, biglycan enhances the expression of pro-inflammatory mediators correlated to gastric cell necroptosis (Guo et al. 2019).

In colon cancer cells, the downregulation of biglycan was correlated to apoptosis. Interestingly, an abrogation of the p38 signaling pathway with SB203580 reversed the enhancement of apoptosis in biglycan-deficient cells (Xing et al. 2015a, b).

On the other hand, biglycan was shown to protect neuroblastoma cells from NO-induced cytotoxicity by attenuating autophagy-dependent AMPK–mTOR signaling as well as the intracellular levels of ROS (Chen et al. 2020a, b).

Lumican-overexpressing B16F1 melanoma cells exhibit attenuated anchorage-independent proliferation and the ability to invade the ECM gel. Indeed, B16F1 cells

presented an induction or an increase of apoptosis in lumican presence (Vuillermoz et al. 2004).

Stromal lumican was demonstrated to decrease hypoxia-inducible factor-1α (HIF1α) expression and activity via Ak correlated to increased pancreatic cancer cell apoptosis (Li et al. 2014). Moreover, lumican was shown to initiate a quiescent pancreatic cancer state, characterized among others with apoptosis (Li et al. 2017).

The importance of cancer-stroma interactions is highlighted by a recent study showing that hypoxia induces autophagy in pancreatic cancer stellate cells, which results in AKMP/TOR/p70S6K/4EBP signaling pathway-mediated protein degradation and synthesis inhibition. Lumican secretion was strongly downregulated in these cells. Indeed, lumican mRNA expression was not altered, verifying that changes in lumican expression were perpetrated at the post-transcriptional level (Li et al. 2019). It is worth mentioning that even though hypoxia-induced autophagy was also determined in ex vivo cultures of patient-derived primary PDAC xenograft cancer cells were not affected (Li et al. 2019). The pancreatic stellate cells have been attributed significant roles in paracrine signaling, metabolism, and tumor-immunology of pancreatic ductal carcinoma (Fu et al. 2018).

1.4 Biglycan: A Regulator of Tumor-Associated Angiogenesis

Overexpression of biglycan in colon cancer cells strongly upregulated their VEGF expression and enhanced the angiogenesis and growth of the tumor xenograft. Indeed, biglycan was suggested to be a promising target for cancer antiangiogenic therapy (Xing et al. 2015a, b). Furthermore, biglycan secreted by gastric cancer cells binds to its TLR2 and TLR4 receptors on endothelial cells. This was correlated to increased endothelial cell VEGF production, migration, and tube formation (Hu et al. 2016).

A significant breakthrough was identifying bidirectional interactions between the tumor cells and the tumor endothelial cells (TECs) that exhibit discrete phenotypes compared to normal counterparts. Biglycan secreted by TECs has been characterized as an autocrine angiogenic factor of tumor endothelial cells (Yamamoto et al. 2012). Notably, tumor endothelial cells isolated from tumors with high metastatic potential were shown to promote the metastasis of the tumor cells with lower aggressivity via epigenetic dysregulation of biglycan (Maishi et al. 2016). Indeed, when biglycan was knocked out in the stroma of E0771 breast cancer-bearing mice, their metastasis to the lung was inhibited. Furthermore, the downregulation of biglycan in the tumor stroma also impaired tumor angiogenesis and normalized tumor vasculature by repressing tumor necrosis factor-α/angiopoietin 2 signaling.

On the other hand, lumican facilitates endothelial cell apoptosis through Fas-dependent signaling. Indeed, lumican-overexpressing murine fibrosarcoma (MCA102) and pancreatic adenocarcinoma (Pan02) cells exhibited attenuated

in vivo growth compared to their lumican nonexpressing counterparts (Williams et al. 2010).

1.5 Effects of Biglycan and Lumican on Chemoresistance

Biglycan was shown to be overexpressed in colon cancer stem cells and induce the activation of the NF-κB pathway, contributing to colon cancer cell chemotherapy resistance. In addition, biglycan attenuated the expression of pro-apoptotic markers and facilitated the activation of the NF-kβ pathway (Liu et al. 2018). Furthermore, the overexpression of biglycan induced rapamycin resistance in WERI-Rb-1 retinoblastoma cells through activating the PI3K/Akt/NF-kappaBaxis (Fang et al. 2019). Mitz et al. suggest that tumor-driven alterations in the osteoid contribute to the development of chemoresistance in osteosarcoma and characterize biglycan as one of the differentially expressed genes (Mintz et al. 2005).

On the other hand, this SLRP was determined to be one of the 10 ECM proteins overexpressed in chemo-sensitive tissue compared to the chemo-resistant tissue (Pan et al. 2009). Biglycan was shown to revert the tumor microenvironment in breast cancer (Moreth et al. 2010).

Notably, chemotherapy agents increased the secretion of lumican in PDAC cells, which was correlated to the extent of response. In various PDAC models, including cell lines, patient-derived xenografts, and lumican knockout mice, lumican was found to enhance the anticancer chemotherapy effect (Li et al. 2016). Specifically, chemotherapeutic agents in PDAC cells facilitate autophagosome formation and enhance LC3 expression through the ROS-mediated AMP-activated kinase (AMPK) signaling pathway. Lumican attenuates AMPK activity, abrogating the protective mechanism of chemotherapy-induced autophagy in in vitro and in vivo PDAC models (Li et al. 2016).

1.6 SLRPs as Cancer Therapy Targets

Considering the many-faceted roles of biglycan and lumican in carcinogenesis, the modulation of their activities are likely targets of anticancer therapies. First steps have been taken in this direction. Thus, biglycan was shown to be the target of celastrol, a triterpene component of Chinese medicine (Guo et al. 2019). Indeed, celastrol in a biglycan-dependent manner activated receptor-interacting protein 1 and 3 (RIP1 and RIP3) and subsequently promoted the translation of mixed-lineage kinase domain-like (MLKL) from the cytoplasm to plasma membrane inducing necroptosis in HGC27 and AGS gastric cancer cells. Moreover, celastrol attenuated the secretion of pro-inflammatory cytokines TNF-α and IL-8 in HGC27 and AGS cells, which was counteracted by the overexpression biglycan. Notably, biglycan has been indicated to connect innate and adaptive immunity through TLR2/4

downstream signaling established to control neutrophil, T and B lymphocyte, macrophage, and dendritic cell activities (Babelova et al. 2009; Zeng-Brouwers 2014; Moreth et al. 2014). Indeed, an increasing amount of data suggests that the activation of TLR2/TLR4 downstream signaling stimulates the secretion of inflammation-associated cytokines by tumor and stromal cells facilitating tumor cell growth (Schaefer and Iozzo 2012; Farnebo et al. 2015; Dajon et al. 2017).

1.7 Conclusions

In summary, biglycan and lumican are significant mediators of carcinogenesis with the ability to modulate all hallmarks of cancer, including cell growth, migration, invasion, apoptosis, immunological response, and angiogenesis.

Lumican is suggested to be both promote cancer development and exhibit antitumor properties. Indeed, increasing data indicate that its role depends on the tumor origin tissue and the stage of the disease. Furthermore, it has been suggested that by regulating tumor cell behavior, lumican can predict the response to radiotherapy and modulate chemotherapy.

On the other hand, biglycan is found to be overexpressed by most solid tumors, and its expression was positively correlated to disease progression. Furthermore, biglycan seems to promote tumor-associated inflammation and facilitate tumor-related angiogenesis. In some tumor types, such as osteosarcoma, biglycan was identified as a negative prognostic biomarker for chemotherapy response. Taking into account that the TME is a composite network of different cell types, biosignaling mediators, and numerous ECM components, with the capability to regulate the biological functions of both tumor and stroma cells cell determining the role of discrete components is vital. The novel data on the multifaceted roles of these SLRPs could potentially be translated into valid therapeutic options. Therefore, further understanding of the biology of the TME is obligatory for the successful targeting of these SLRP as therapeutic strategies.

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Chapter 2
The Role of Decorin in Cancer

Kornélia Baghy, Andrea Reszegi, Zsolt Horváth, and Ilona Kovalszky

Abstract The dynamic interactions between the tumor cells and their microenvironment consisting of stroma cells and extracellular matrix play a crucial role in cancer development. Decorin, a chondroitin sulfate/dermatan sulfate proteoglycan of the SLRP family is expressed in the stroma of cancers with various origin. Decorin was originally considered as a structural component of the ECM, but research of the last few decades discovered its essential roles in fibrotic pathologies, inflammation, and cancer. The initial observation of its being a natural inhibitor of TGFβ was followed by decorin's ability to counteract with the activity of several receptor tyrosine kinases such as EGFR, Met, IGF-IR, VEGFR, and PDGFR. Besides modulating the RTK signaling, decorin induces the degradation of these receptors by initiating their caveosomal internalization. Decorin also provokes cell cycle arrest and apoptosis, and exerts antimetastatic and antiangiogenic effects. The discovery of decorin's action in conserved catabolic processes, namely endothelial cell autophagy and tumor cell mitophagy further broadened the palette of its antitumor abilities. Therefore, in spite of the difficulties in mass producing the proteoglycan, decorin represents a promising tool against cancer, especially against those, employing altered RTK signaling.

2.1 Introduction

The decorin proteoglycan is the prototype and best-characterized member of the small leucine-rich proteoglycan (SLRP) family. Its name refers to its strong interaction with collagen fibers appearing as "decoration" of fibrils (Reese et al. 2013; Zhang et al. 2009; Chen et al. 2014; Iozzo and Schaefer 2015; Chen and Birk 2013). The gene of decorin was 1st cloned in 1986; at that time the proteoglycan was

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considered only as a structural constituent of the extracellular matrix (ECM) (Krusius and Ruoslahti 1986). With time, decorin was proved to exert a plethora of cellular functions such as inhibiting proliferation, migration, differentiation, metastasis formation, modulating angiogenesis or inflammatory responses (Yamaguchi and Ruoslahti 1988; Ruoslahti and Yamaguchi 1991; Border et al. 1992; Jarvinen and Prince 2015). These first reports on the antitumor effects of decorin paved the way for further and still ongoing studies to seize the opportunity of its therapeutic utilization against cancer (Sofeu Feugaing et al. 2013).

Mammalian decorin is highly conserved across the species. This proteoglycan is mainly produced by fibroblasts, myofibroblasts, macrophages, and smooth muscle cells (Zhang et al. 2018). Decorin consists of a 42 kDa protein core containing a central domain with 12 leucine-rich repeats (LRR) flanked by cysteine-rich regions and an N-terminal attachment site for a single glycosaminoglycan (GAG) chain, that can be either chondroitin or dermatan sulfate (Krusius and Ruoslahti 1986; Mann et al. 1990). The 12 LRRs form a horseshoe or banana shape (Scott 1996; Weber et al. 1996; Brown et al. 2002; Scott et al. 2004), and this unique structure can interact with a variety of proteins and is responsible for the diverse biological functions of decorin (Neill et al. 2016) (Fig. 2.1). Monomeric decorin represents the active form of the molecule, but the proteoglycan forms homodimers in physiological solutions (Brown et al. 2002; Islam et al. 2013; Goldoni et al. 2004). Dimerization, which seems reversible, prevents the interactions with binding partners by blocking the central domain (Islam et al. 2013). Monomeric decorin acts as a soluble paracrine factor that modulates several downstream signaling pathways (Gubbiotti et al. 2016). In addition, decorin is a substrate of proteolytic enzymes called matrix metalloproteases (MMP-2, MMP-2, MMP-7, MT1-MMP) and BMP-1 peptidase that leads to the cleavage, thus inactivation of the molecule (Mimura et al. 2009; von Marschall and Fisher 2010).

The biological functions of decorin were mostly elucidated by the identification and characterization of mutations occurring in patients suffering of genetic diseases and decorin-deficient mice. Decorin-deficient mice are fertile and display skin fragility phenotype with loosely packed collagen networks that resemble the human Ehlers-Danlos syndrome (Corsi et al. 2002). Mutations in the decorin gene result in connective tissue disorders like congenital stromal corneal dystrophy (Bredrup et al. 2005; Seidler 2012), characterized by the impairment of corneal opacity and vision. The heterozygous mutation affects the ear region of the molecule causing bilateral corneal opacities in patients (Zhang et al. 2009; Pietraszek-Gremplewicz et al. 2019).

In this chapter, we will review the cellular and molecular roles of decorin in cancers including tissue distribution, signaling mechanisms, and also therapeutic applications.

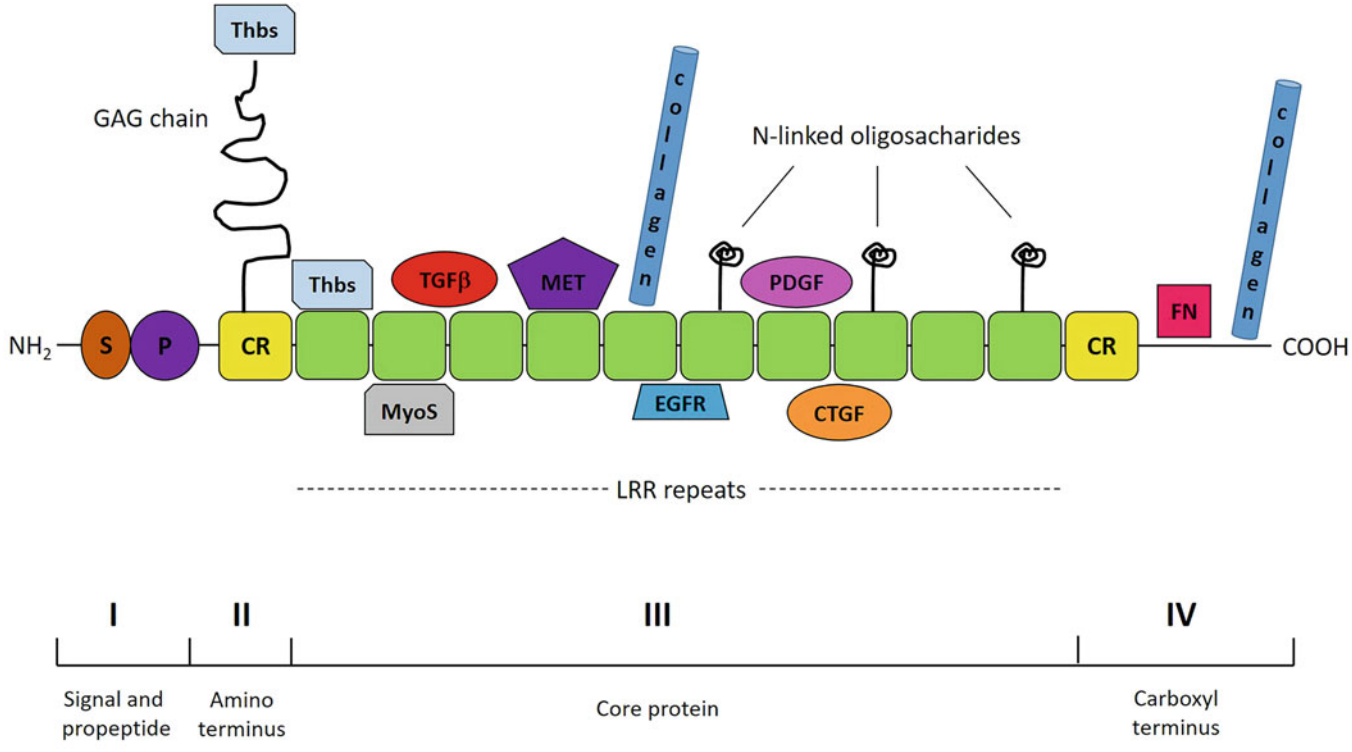

Fig. 2.1 The schematic structure and interacting partners of decorin. The proteoglycan has four domains indicated as I-IV. The core protein contains tandem leucine-rich repeats (LRR) flanked by cysteine-rich regions (CR). The molecule bears one chondroitin sulfate/dermatan sulfate glucosaminoglycan (GAG) chain and three N-linked oligosaccharides. Decorin interacts with a large number of molecules including growth factors such as TGFβ, PDGF, CTGF, receptor tyrosine kinases like EGFR and Met), myostatin (MyoS), thrombospondin, and structural components of the ECM e.g. collagens or fibronectin. As shown in the figure, decorin displays different binding sites to these signaling molecules, which suggest simultaneous modulation of different pathways for curbing tumorigenesis

2.2 The Uprise of Decorin as an Antitumor Agent

2.2.1 Genetic Evidences

The first evidences for the tumor suppressor property of decorin emerged from experimental mouse models where the decorin gene was unconditionally ablated (Danielson et al. 1997). Decorin knockout mice developed spontaneous intestinal tumors when fed with high-fat diet (Bi et al. 2008). In this model, the lack of decorin led to disturbed intestinal maturation as a result of reduced cell differentiation and increased proliferation. As underlying mechanisms, downregulation of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, intestinal trefoil factor, and E-cadherin, as well as the upregulation of β-catenin signaling were identified (Bi et al. 2008). These revealed signaling events were the pioneers paving the way to further studies to uncover the antitumor mechanisms exhibited by decorin. Simultaneous disruption of both decorin and p53 genes resulted in the formation of aggressive T-cell lymphomas and premature death of these mice (Iozzo et al. 1999a). Ablation of decorin gene also resulted in enhanced tumor incidence and tumor count in livers of mice exposed to hepatocarcinogens (Horvath et al. 2014). In a model of colitis-associated cancer, the intestinal endothelium of decorin knockout mice showed an increased protein levels of Snail, Slug, Twist, and MMP2, factors associated with epithelial-mesenchymal transition. In addition to the previous, in colitis-associated tumors, ICAM-1 expression was also significantly elevated in $Dcn^{-/-}$ mice compared to wild-type animals (Mao et al. 2021).

2.2.2 Decorin Expression in Cancers

To understand the biological role of decorin in cancer, we have to discuss its expression patterns and localization within tumors. Only few studies exploring decorin expression in human tumors are available vs. in vitro investigations. However, a general phenomenon based on the available reports can be highlighted, namely that decorin expression tends to be downregulated in the parenchyma of advanced tumors of various origin, but sometimes seen with overexpression in the stroma. In human breast cancer, decorin expression is inhibited both at the mRNA and protein level when compared to either normal or non-tumorous adjacent tissues (Leygue et al. 2000). Similar observations on decorin downregulation were detected in other malignancies, such as endometrial (Smid-Koopman et al. 2000), lung (Campioni et al. 2008), and ovarian (Nash et al. 2002) cancers. In line with the previous reported data, a reduced decorin expression was associated with poor prognosis in node-negative invasive breast carcinoma and soft tissue tumors (Troup et al. 2003; Matsumine et al. 2007). Analyzing the Human Protein Atlas database, a significant reduction in decorin levels was revealed in the stroma of bladder, breast, cervical, colon, kidney, pancreas, prostate, ovary, rectal, skin,

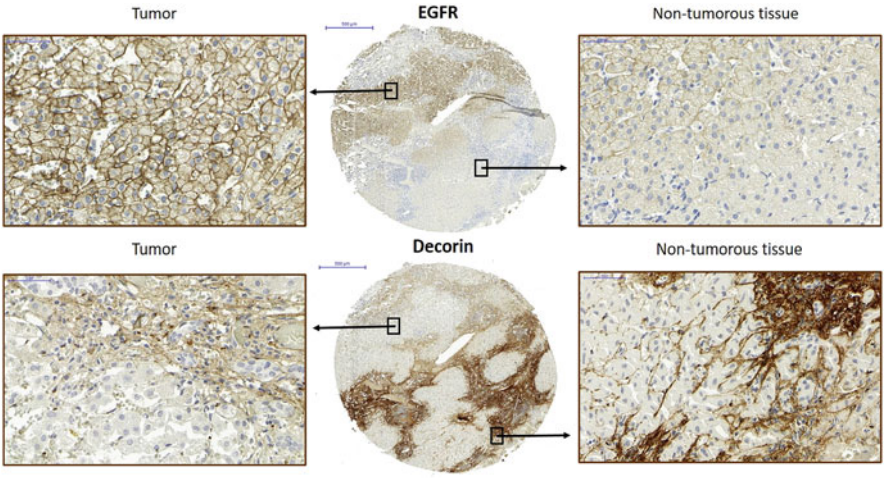

Fig. 2.2 Alternating expression of decorin and EGFR in tumorous liver tissues. In liver cancer, decorin expression is markedly reduced, even absent compared to the connective tissue of the non-tumorous liver tissue. However, EGFR, a known binding partner of decorin exerts exactly the opposite expression pattern suggesting a functional link between the two molecules. Identical tissue microarray cores of human material were simultaneously stained with decorin and EGFR antibodies

stomach, and testis tumors (Bozoky et al. 2014; Neill et al. 2015). Decorin expression was abolished in the stroma of low- and high-grade urothelial carcinoma, while elevated decorin level was detected in the submucosa and deep tumor stroma (Iozzo et al. 2011). In a colon carcinoma-liver metastasis model, normal colon exerted the highest decorin expression, but the proteoglycan level was also abundant in the stroma of the primary tumor. In contrast, liver metastasis of the same tumors generally contained reduced levels of decorin, which were associated to the aggressiveness of the disease (Reszegi et al. 2020). Decreased decorin expression was described in multiple myeloma and monoclonal gammopathy of undetermined significance (Kristensen et al. 2013), in esophageal squamous cell carcinoma (Wu et al. 2010), and in some cases of colon carcinoma (Nyman et al. 2015). Several studies reported the total absence of decorin within the tumor parenchyma in various tumors such as urothelial, prostate, myeloma, and liver cancer (Li et al. 2008; Sainio et al. 2013; Bostrom et al. 2013; Henke et al. 2012; Horvath et al. 2014; Duncan 2013) (Fig. 2.2).

In general, in tumor cells as well as in non-tumorous connective tissues decorin is missing, or its expression is very low. However, the tumorous stroma may contain high levels of decorin as reported in human colon (Iozzo et al. 1982, 1989; Iozzo and Wight 1982) and breast cancers (Santra et al. 1995; Brown et al. 1999). In addition, decorin expression is also highly enhanced in the ECM of pancreatic carcinoma, mainly detected at the border of tumorous areas. As source of the excessive decorin deposition, pancreatic stellate cells were identified (Skandalis et al. 2006; Sofeu

Feugaing et al. 2013; Koninger et al. 2004), while decorin expression was completely absent in pancreatic cancer cells.

It is a feasible hypothesis that the stromal accumulation of this proteoglycan is still able to hinder tumor growth by acting as a physical barrier similar to that seen in desmoplastic reactions. In line with that, as a paracrine inhibitor, stromal decorin can also block the action of receptor tyrosine kinases located in the membranes of tumor cells.

To add some spice to what already discussed, high decorin mRNA expression was found during the early stages of tumorigenesis as a result of transcriptional analysis of tumor progression. In line with that high decorin mRNA expression was detected in hemangiomas, while decorin production was completely abolished in malignant vascular sarcomas (Salomaki et al. 2008). The same phenomenon was reported in case of patients with B-cell chronic lymphoid leukemia (CLL), as high decorin mRNA levels were detected in early stages and with non-progressive CLL, while it was suppressed in advanced stages and in patients with the aggressive type of the disease (Campo et al. 2006). It seems conceivable that the loss of decorin expression correlates with tumor progression and aggressiveness, and could be utilized as a marker of early and late-stage cancer.

2.2.3 Signaling in Cancers

2.2.3.1 Interaction with TGF-β and Other Growth Factors

The **transforming growth factor-β (TGF-β)** (Yamaguchi et al. 1990) was the first growth factor identified as an interacting partner of decorin. The decorin protein core does not distinguish among the growth factor isotypes, as it is able to bind to TGF-β1, TGF-β2, and TGF-β3 (Hildebrand et al. 1994). The interaction of decorin with TGF-β results in attenuated proliferation and spreading of several TGF-β – dependent tumor cell lines (Jarvinen and Prince 2015; Yamaguchi et al. 1990). As a mechanism of action, it was proposed that decorin traps TGF-β before it binds to its receptors and anchors it to the collagens of the ECM (Jarvinen and Ruoslahti 2013). Several in vitro and in vivo studies confirmed that decorin inhibits TGF-β signaling pathways (Gubbiotti et al. 2016; Abdel-Wahab et al. 2002; Kolb et al. 2001; Baghy et al. 2011). The decorin-TGF-β complex is inactive, and such binding results in hindered signaling via Smad2, Smad3, and Erk1/2, and attenuates fibrogenesis and inflammation (Baghy et al. 2012). Intriguingly, TGF-β is able to inhibit decorin expression at the transcriptional level as observed in fibroblasts (Mauviel et al. 1995), suggesting a feedback mechanism responsible for the balance in matrix deposition. Another cellular process linked to TGF-β is the regulation of inflammation. Due to its ability to reduce inflammation and fibrosis, decorin was proposed as a physiological TGF-β-inhibitor that limits the duration of TGF-β responses in inflammation and tissue repair (Border et al. 1992; Jarvinen and Ruoslahti 2013; Jarvinen and Prince 2015). As the function of TGF-β in cancer is quite controversial and

highly depends on the cellular context (Ikushima and Miyazono 2010), neutralization of the growth factor either by decorin or other substances as a potential antitumor strategy needs careful evaluation (Baghy et al. 2020).

In addition to TGF-β, decorin also binds to and inhibits two other members of the TGF-β superfamily, namely myostatin and activin C. In case of **myostatin**, interaction with decorin curbs myostatin-provoked inhibition of myofiber growth leading to improved muscle regeneration (Kanzleiter et al. 2014; Miura et al. 2006; Zhu et al. 2007). In addition, myostatin was reported to play a crucial role in the process of cancer cachexia, and its blocking by antagonists has a beneficial effect on survival (Argiles et al. 2017; Miyamoto et al. 2016; Sharma et al. 2015). These observations may pave the way for decorin to counteract with cancer-related wasting by antagonizing myostatin. The interaction of decorin and the oncogenic **activin-C** leads to endocytosis of the complex with subsequent degradation. The event culminates in reduced proliferative and migration capacity of colorectal carcinoma cells in vitro and in vivo (Bi et al. 2016).

Another two interacting partners of decorin belong to the connective tissue growth factor (CTGF) family. Decorin binding inhibits the action of **CTGF** (Vial et al. 2011; Gubbiotti et al. 2016) a key player in several cellular processes such as chemotaxis, proliferation and differentiation, fibrosis, regulation of ECM production, and modulation of inflammation (Lipson et al. 2012; Luft 2008; Kular et al. 2011). Interestingly, CTGF itself induces decorin expression, its own inhibitor, and at the same time is tightly regulated by TGF-β suggesting a fine-tuned autoregulation process in the system (Vial et al. 2011). The other CTGF family member, known to bind to decorin is the **Wnt-inducible signaling pathway protein-1** (**WISP-1**, alias CCN4), a known oncogene in a variety of cancers (Nivison and Meier 2018). Decorin binds to WISP via its dermatan sulfate GAG chain modulating Wnt signaling (Neill et al. 2012b; Desnoyers et al. 2001), and at the same time, it is known to inhibit **β-catenin** (Bi et al. 2008; Young et al. 1998), although a direct link between the two mechanisms has not been verified yet.

2.2.3.2 Structural Components as Binding Partners

A less explored, but not less important binding partners of decorin, are the structural components within the ECM including several types of collagens, tenascin X (Elefteriou et al. 2001) and elastin (Reinboth et al. 2002). In addition, complexes of matrilin-1 and decorin connect collagen VI microfibrils to aggrecan or collagen type II (Wiberg et al. 2003). These studies emphasize that decorin has a pivotal role in the regulation of ECM structure and may be an active participant in the desmoplastic reactions in the tumor stroma, a defensive mechanism of the host tissue against cancer (Neill et al. 2012b).

2.2.3.3 Decorin Acts as a pan-RTK Inhibitor

A whole new era of the antitumor action of decorin started with the striking observations that decorin directly interacts with a variety of receptor tyrosine kinases (RTKs). Proven with a set of studies on decorin's tumor inhibitory action via RTKs and subsequent signaling pathways led to the consideration of "the guardian from the matrix" (Neill et al. 2012b).

Epidermal growth factor receptor (EGFR) was the first RTK identified to interact with decorin (Fig. 2.2). In details, binding of the proteoglycan to EGFR at a partially overlapping site with EGF resulted in transient activation of EGFR followed by caveolin-1 mediated internalization and degradation of the receptor (Iozzo et al. 1999b; Moscatello et al. 1998; Santra et al. 2000, 2002; Zhu et al. 2005; Neill et al. 2012b). As a consequence, decorin-EGFR interaction culminated in cell cycle arrest, apoptosis, angiostasis, and prolonged suppression of oncogenes (Gubbiotti et al. 2016; Neill et al. 2012b, 2015). Downstream of EGFR, decorin enhanced the action of MAPK pathway and intracellular Ca^{2+} release (Seidler et al. 2006; Csordas et al. 2000), induced the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and the level of cleaved caspase-3 in A431 squamous carcinoma cell line (Seidler et al. 2006). The blocking action of decorin on EGFR and its downstream signaling was further corroborated by other in vitro and in vivo studies. In A549 lung carcinoma cells, inhibited EGFR action by decorin overexpression resulted in induced p53 and $p21^{WAF1/CIP1}$ expression leading to cell cycle blockade in G1 and apoptosis (Liang et al. 2013). Vice versa, genetic ablation of decorin enhances EGFR, and subsequently ERK1/2 phosphorylation in experimental hepatocarcinogenesis models (Horvath et al. 2014).

Besides EGFR, ErbB2 and ErbB4 (other members of the ErbB family) were found to interact with decorin (Goldoni et al. 2008; Gubbiotti et al. 2016; Goldoni and Iozzo 2008). Here, decorin-provoked growth arrest and stimulated differentiation occurred due to the fact that ErbB4-decorin binding hampered the dimerization of ErbB4 with ErbB2 in breast carcinoma cells (Goldoni et al. 2008).

The Met receptor tyrosine kinase (hepatocyte growth factor receptor, scatter receptor) is another important and well-described binding partner of decorin (Goldoni et al. 2009) (Fig. 2.3).

Similarly to EGFR, the interaction between decorin and Met induces autophosphorylation of the receptor leading to its proteasomal degradation (Goldoni et al. 2009; Neill et al. 2015). Inhibition of Met activity by decorin results in suppressed downstream signaling, including selective degradation of β-catenin and Myc oncoproteins, ending up with inhibited tumor growth (Buraschi et al. 2010; Goldoni et al. 2009; Neill et al. 2015). The failure of nuclear translocation of β-catenin and the subsequent transcriptional repression of its target gene MYC initiates a series of processes (Hanahan and Weinberg 2011; Buraschi et al. 2010; Horvath et al. 2019). Myc is a key transcription factor playing crucial role in the coordination of a variety of pathways that stimulates cell proliferation. Myc targets AP4, a known transcriptional repressor of the $p21^{WAF1/CIP1}$ cyclin dependent kinase

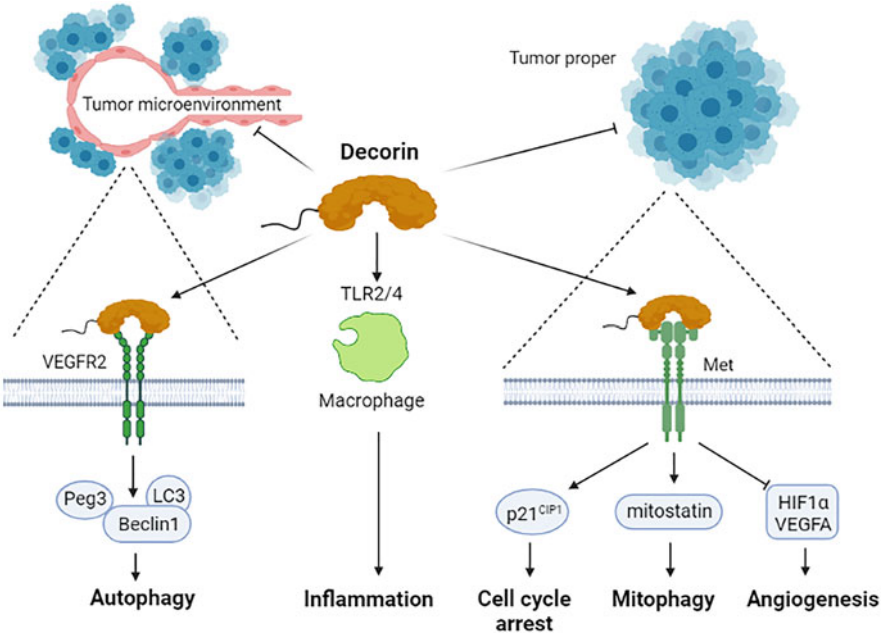

Fig. 2.3 Comprehensive illustration of interactions and signaling events provoked by decorin. The proteoglycan exerts an inhibitory effect on both the tumor microenvironment and the tumor itself. On the surface of endothelial cells, decorin blocks the action of VEGF receptor leading to autophagy via Peg3, beclin, and LC3. Within the tumor microenvironment, decorin binds to TLR2 and TLR4 on the surface of macrophages inducing inflammatory processes. Among other RTKs, decorin interacts with the Met receptor in the membrane of tumor cells. This interaction inhibits the activity of Met, which subsequently leads to cell cycle arrest via $p21^{CIP1}$ and tumor cell mitophagy via mitostatin. By hindered HIF1α and VEGF production, decorin exerts anti-angiogenic actions. (Created with BioRender.com)

inhibitor (Jung et al. 2008). Indeed, decorin was reported to block cell cycle via inducing $p21^{WAF1/CIP1}$ as a key mechanism for curbing tumorigenesis in many cancer models (Iozzo and Sanderson 2011; Neill et al. 2012b). Moreover, inhibition of Met by decorin leads to the decrease of two proangiogenic factors namely VEGFA and HIF-1α via stimulation of the angiogenesis inhibitor TIMP-3 (Neill et al. 2012a).

Since the original observation of decorin's blocking action on EGFR, several other RTKs have joined the expanding family of decorin's interacting partners. The proteoglycan can regulate the insulin signaling pathway via several mechanisms. It modulates the activity of the **insulin-like growth factor receptor-1 (IGF-1R)** and **insulin receptor (IR)** and their ligands (Morcavallo et al. 2014; Morrione et al. 2013; Schonherr et al. 2005; Baghy et al. 2013). In contrast to the caveosomal endocytosis and subsequent degradation of EGFR and Met upon decorin binding, here the proteoglycan hinders IGF-1R activity via IRS1 and IGF-1. Moreover, decorin-IGF-1 interaction prevents translocation of IGF-1R to caveosomes (Iozzo

et al. 2011; Neill et al. 2012b; Schaefer et al. 2007; Schonherr et al. 2005). However, the action of decorin on IGF-1R signaling is contradictory. It seems that decorin antagonizes IGF-1R only in cancers, but enhances IGF-1R function in normal tissues (Neill et al. 2016; Morrione et al. 2013). To further nuance the mechanism, comparison of four hepatoma cell lines revealed that IGF-1R as well as IR can be either enhanced or inhibited even in tumor cell lines with the same tissue origin (Horvath et al. 2019). In addition, temporal change of receptor activation was also observed, as initial phosphorylation of IGF-1R and IR upon decorin exposure declined rapidly, and attenuated receptor activity was observed after 2 days. These reports emphasize that the role of decorin in regulating IGF-1R is complex and is dependent on unique characteristics of the tumor (Horvath et al. 2019).

Similarly to IGF-1R, the inhibitory action of decorin on **platelet-derived growth factor receptor-α (PDGFRα)** takes place via direct sequestration of its ligand PDGF (Baghy et al. 2013; Horvath et al. 2014) leading to inhibited downstream signaling in liver cancer, and hindered cellular migration and the development of intimal hyperplasia after balloon angioplasty (Nili et al. 2003).

In addition decorin is known to interact with the **vascular endothelial growth factor receptor-2** (VEGFR2, see also later) (Khan et al. 2011; Lala et al. 2012) and the macrophage stimulating protein receptor (MSPR/RON) (Horvath et al. 2014).

2.2.3.4 Intracellular Signaling Pathways

Downstream of RTK receptors discussed above, decorin effectively hinders several pathways involved in proliferation, migration, angiogenesis, or survival such as the Ras/MEK/ERK and PI3K/Akt/mTOR representing the main and widely studied pathways in a variety of cancers (Whittaker et al. 2010; Villanueva et al. 2007). In addition, the GSK-3β is a key molecule that serves as a node linking several signaling pathways (e.g. originated from Wnt and RTKs) and is known to be inactivated by Akt (Grimes and Jope 2001; Jacobs et al. 2012). The mechanisms by which decorin inhibits β-catenin and Myc, and the role of $p21^{WAF1/CIP1}$ in G1/S phase cell cycle blockade have already been discussed in this chapter. In addition to $p21^{WAF1/CIP1}$, decorin is able to induce the expression of other cyclin dependent kinase inhibitors namely $p27^{KIP1}$ (Xaus et al. 2001; Bi et al. 2008, 2012), $p15^{INK4b}$ (Liu et al. 2016) and $p57^{KIP2}$ (Hamid et al. 2013), resulting in attenuated cell cycle progression.

Besides its capability to arrest cell cycle at G1/S transition, decorin was described to block G2/M transition in HEP3B hepatoma cells (Horvath et al. 2019). This particular cell line harbors deleterious mutations of both p53 and retinoblastoma (Rb) genes (Mitry et al. 1997) that leads to continuous transition from G1 to S as the restriction point is compromised. As a mechanism of action, the researchers identified activated the ATR/Chk1/Wee1 signaling upon decorin exposure, blocking the cell cycle at the G2/M via phosphorylation of CDK1. In parallel, high phospho-AKT levels downstream of IR and IGF-1R were detected along with decreased Cdc25A phosphatase expression strengthening the novel mechanism described (Horvath

et al. 2019; Benada and Macurek 2015). The genes of Rb and p53 tumor suppressor proteins are among the top most frequently mutated genes in tumors. The discovery that decorin is capable to exert its antitumor action even in cells lacking functional p53 and Rb proteins further reinforces its utilization against cancers.

In summary, the interactions of decorin with RTKs and other molecules trigger countless changes in cell signaling pathways culminated in hindered tumorigenesis (Gubbiotti et al. 2016). Thus, the introduction of decorin in tumors dependent on one or more of the proteoglycan's interacting partners may be beneficial (Goldoni et al. 2008; Bostrom et al. 2013; Horvath et al. 2014; Neill et al. 2015; Reed et al. 2005). However, it must be kept in mind that the effect of decorin is highly cell type-specific.

2.2.4 Inflammation, Immunomodulation

Several studies evidenced that decorin as well as its relative molecule biglycan is capable of regulating inflammatory and innate immune responses via TLR2 and 4 in tissue stress and injury (Merline et al. 2011; Neill et al. 2016; Schaefer 2014; Moreth et al. 2014; Zeng-Brouwers et al. 2014). Direct interaction between the proteoglycan and TLR2/4 on macrophages results in transient activation of MAPK and NFκB pathways with consequent secretion of tumor necrosis factor-α (TNFα) and IL-12p70 inflammatory molecules (Moreth et al. 2010) (Fig. 2.3). On the top of that, decorin indirectly modulates inflammation by inhibiting the action of TGF-β1, and consequently microRNA-21 (miR-21), a process that liberates macrophage-derived proinflammatory programmed cell death protein 4 (PDCD4) from translational repression (Merline et al. 2011). This mechanism leads to a proinflammatory tumor microenvironment by suppressing anti-inflammatory molecule IL-10 (Neill et al. 2012b, 2016; Gubbiotti et al. 2016; Zhang et al. 2018; Merline et al. 2011). Decorin is able to sustain the inflammatory state by enhancing CCL2 production that attracts mononuclear cells to the site of injury (Koninger et al. 2006). Decorin is also able to modulate the attachment of immune cells. By interacting with class A scavenger receptors, it enhances macrophage adhesion to the matrix (Santiago-Garcia et al. 2003). On the contrary, decorin directly inhibits the adhesion of polymorphonuclear leukocytes to the endothelium, and in parallel decreases the expression of ICAM-1 and the anti-inflammatory syndecan-1 (Seidler et al. 2011).

Generally, proinflammatory tumor microenvironment is known to retard tumor growth, thus unveiling the role of decorin in regulating inflammation is of crucial importance (Gubbiotti et al. 2016; Zhang et al. 2018; Moreth et al. 2012; Schaefer and Iozzo 2012). However, decorin was also reported to inhibit inflammatory and chemotactic genes of immune response (Buraschi et al. 2012). Decorin deficiency always results in a pro-inflammatory and pro-fibrotic phenotype as reported in several in vivo models; furthermore, treatment with exogenous decorin suppresses inflammation in experimental trials (Jarvelainen et al. 2006, 2009; Border et al. 1992; Jarvinen and Ruoslahti 2010, 2013). In addition, tumor necrosis factor-α

(TNF-α), a key proinflammatory cytokine, is known to interact with, and sequestered away by decorin, which prevents its binding to receptors (Tufvesson and Westergren-Thorsson 2002).

What determines whether decorin exerts pro or anti-inflammatory action is still far from being understood, and additional studies are to resolve the contradictions. However, it was reported that neither the protein core nor the GAG chain alone is capable to induce TNFα and IL-12p70 (Merline et al. 2011). In another study, it was the core protein that attenuated the expressions of anti-inflammatory genes (Buraschi et al. 2012). These observations give raise to the possibility that the protein core and the glycanated decorin displays different actions regarding immune responses (Zhang et al. 2018).

In addition, decorin can also be inactivated by proteases secreted by inflammatory cells. Decorin as a member of damage associated molecular patterns (DAMPs) may be recognized by pattern recognition receptors such as Toll-like receptor-2 (TLR2) and TLR4 inducing an inflammatory response (Schaefer 2014).

2.2.5 Autophagy, Mitophagy, and Angiogenesis

Decorin was discovered to indirectly induce autophagy of vascular endothelial cells, a process that attenuates tumor spread and metastasis formation via transcriptionally enhancing Peg3 (paternally expressed 3) tumor suppressor (Neill et al. 2016, 2021; Zhang et al. 2018; Buraschi et al. 2012). PEG3 is known to be hypermethylated thus inactive in a variety of tumors (Kuroiwa et al. 1996; Yamaguchi et al. 2002; Kohda et al. 2001; Dowdy et al. 2005), and can inhibit the Wnt/β-catenin signaling pathway, partly accounting for the decorin's antitumor action (Jiang et al. 2010; Neill et al. 2015). As a mechanism of action, decorin interacts with and activates VEGFR2 receptor in the membrane of endothelial cells and induces AMPK/Vps34 signaling pathway with concurrent inhibition of the anti-autophagic Akt/mTOR pathway (Schaefer and Dikic 2021; Alers et al. 2012; Goyal et al. 2014) (Fig. 2.3). In the process, decorin induces beclin-1 and the microtubule-associated protein light chain 3 (LC3) via Peg3 culminating in the formation of autophagy precursor complexes (Neill et al. 2013, 2015). In addition, decorin curbs the inhibitory Bcl-2/beclin-1 complex formation (Pattingre and Levine 2006). Importantly, beclin-1 can be inactivated by EGFR and Akt signaling leading to chemoresistance and suppression of autophagy (Wang et al. 2012; Wei et al. 2013). This process may represent a general scheme as downstream signaling of several RTKs overlap. Besides inducing autophagy on endothelial cells, decorin compromises their attachment, migration and differentiation and sprouting mainly via inhibition of VEGF expression (Grant et al. 2002; Neill et al. 2012a; Buraschi et al. 2013).

Besides endothelial autophagy, decorin is able to induce mitochondrial autophagy (mitophagy), thus directly modulate catabolic processes within the tumor (Neill et al. 2016). Decorin promotes mitophagy via induction of the Met receptor that results in enhanced expression and accumulation of the mitostatin

tumor suppressor present in the mitochondrial associated membrane (Neill et al. 2014). Mitostatin is crucial for decorin to trigger mitophagy as depletion of the molecule compromises the inhibition of VEGFA by decorin. As decorin is able to raise Ca^{2+} level with subsequent depolarization of the mitochondria via interaction with EGFR, it is plausible that it is the mitostatin that mediates the process (Neill et al. 2016).

In conclusion, by inducing endothelial autophagy decorin inhibits neoangiogenesis in the tumorous stroma leading to inhibition of spreading and metastasis formation of tumors. In parallel, by interacting with RTKs, decorin enhances mitophagy modulating the catabolic processes in tumor cells (Neill et al. 2020).

2.3 Therapeutic Applications

Since the discovery of decorin's antitumor actions, enormous number of studies have been carried out to investigate the applicability of the proteoglycan as a therapeutic agent. Early studies applied in vivo virus-mediated delivery of decorin and proved its protecting effect in several different tumors. For example, decorin attenuated the growth of colon, lung, squamous cell carcinoma, inhibited invasion and metastasis formation of prostate cancer, osteosarcoma, and breast cancer as well (Reed et al. 2002; Xu et al. 2015; Shintani et al. 2008; Tralhao et al. 2003; Araki et al. 2009; Yang et al. 2015). Decorin gene therapy curbed tumor growth and had a beneficial effect on survival in an expression and time-dependent manner in a glioma model (Ma et al. 2014), and was successfully utilized in experimental models of prostate and pancreas cancer (Hu et al. 2009; Na et al. 2015).

Regarding therapeutic viral vector applications, decorin as a key player in organizing ECM, may have another profound role. As the ECM normally hampers viral spread into the tumor (Zhang et al. 2018; Parato et al. 2005), decorin is able to enhance the penetration of the virus applied (Choi et al. 2010). In parallel, by inhibiting TGF-β decorin counterweights the immunosuppressive tumor microenvironment (Zhang et al. 2018; Zwirner et al. 2010; Oh et al. 2017).

Recombinant decorin was also successfully applied in several in vitro and in vivo studies. In breast cancer both administration of decorin core protein and transfection of DCN cDNA resulted in lessened EGFR activity, enhanced apoptosis, and attenuated the growth of tumors (Csordas et al. 2000; Santra et al. 2000; Goldoni and Iozzo 2008). In line with that, systemic application of decorin led to growth retardation of breast cancer, inhibited metabolism, and decreased the number of lung metastases (Goldoni et al. 2008; Seidler et al. 2006).

Decorin is capable to modulate the effects of certain therapeutic drugs. Decorin exerts a synergistic effect with carboplatin by hindering the proliferation of ovarian cancer cells (Nash et al. 1999). On the other hand, decorin suspended the cytostatic action of carboplatin and gemcitabine in pancreatic tumor cells, but still exerted its antiproliferative action (Koninger et al. 2004).

The development of decorin into a clinical drug is yet to come, in spite of the great number of in vivo antitumor and antifibrotic investigations and preclinical studies. Its production on a large scale may be challenging as the proteoglycan is heterogeneous in size due to its GAG chains. To meet the criteria for human drugs, the recombinant decorin must contain only the core protein responsible for most of decorin's antitumor actions such as for interacting with RTKs and growth factors (Mann et al. 1990; Jarvinen and Prince 2015).

In the last years, researchers went further as to boost decorin's biological activity thus being a more attractive therapeutic candidate. As a model example, a fusion protein containing the decorin core protein and a small peptide named "CAR" was created. This CAR tag (its exact sequence is CARSKNKDC) selectively delivers large amounts of decorin to inflammatory and neoangiogenic vasculature of tumors and regenerating tissues (Jarvinen and Prince 2015; Jarvinen et al. 2015; Jarvinen and Ruoslahti 2010, 2013; Urakami et al. 2011). In addition, this CAR-DCN peptide exerts a more powerful inhibitory effect on the proliferation and spreading of tumors dependent on TGF-β (Jarvinen and Ruoslahti 2007, 2010). Because of its specificity, the CAR peptide seems to be a convenient agent for targeted delivery of therapeutic agents (Gupta et al. 2014a, b, 2015; Nahar et al. 2014; Toba et al. 2014; Urakami et al. 2011). Based on these improvements, the utilization of decorin as a therapeutic drug against cancer and diseases related to inflammation and angiogenesis is well founded (Jarvinen and Prince 2015).

2.4 Conclusions

With the emerging evidences on the important role of the tumor microenvironment in tumorigenesis, molecules of the ECM have gained more and more attention in the past decades. Decorin is the archetype, and the best-studied member of the SLRP family, originally thought to be a structural protein regulating fibrillogenesis. Since then, the proteoglycan was discovered to play essential roles as a signaling molecule in fibrotic diseases, inflammation, and cancer. Research conducted on decorin-null mouse models unveiled that the absence of decorin enhances tumorigenesis. In line with that tumors with different origin generally reduce decorin expression pointing to its tumor suppressor effect.

On the same note, application of decorin either as a recombinant protein or delivered by vectors inhibits tumor development, progression, spreading, and angiogenesis as proved in a vast number of experimental studies. The basis of decorin's antitumor action is the broad range of its interacting partners including growth factors, tyrosine kinase receptors, ECM component that results in mediating downstream signaling pathways. In addition to its negative effect on cell proliferation, migration, and angiogenesis, decorin is able to induce endothelial cell autophagy and tumor cell mitophagy. These novel regulatory mechanisms further broaden the proteoglycan's effect on tumorigenesis.

Despite of the obvious complexity of its biological actions, all of decorin's interactions culminate in inhibition of tumor progression. The antitumor action of decorin appears at multiple levels: 1) directly affecting tumor cells; 2) modifying the tumor-promoting tumor microenvironment. In addition, therapeutic utilization of decorin is on the way; also researches for improved delivery and activity of the proteoglycan, and of coping with production challenges are promising. Hence, we can say that decorin has well-earned the name "a guardian from the matrix," and will serve as a tool in the battle against cancer.

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Chapter 3
The Mystery of Syndecan-1 in Tumor Development and Progression

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Abstract Syndecan-1, a cell surface heparan sulfate proteoglycan, participates in a multitude of cellular processes, and it acts as a "fine tuner of cellular signaling." Syndecan-1 is present in most epithelial tissues and on the cellular level, it is critical for tumor cell differentiation, adhesion, growth, and invasion. Its expression carries valuable information related to the clinical outcome and prognosis of most solid tumors. Despite considerable efforts to decipher its significance in tumor development and progression, current data point toward both inhibitory and promoting effects, sometimes in a contradictory manner. A large body of evidence shows the importance of the aberrant cytoplasmic-, nuclear-, stromal localization of soluble/shed syndecan-1. In this chapter, we highlight the structure and various interactions of syndecan-1 in relation to its cellular localization and its impact on the prognosis of individual tumor types.

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3.1 Structure and Localization of Syndecans

Syndecan-1, the first member of the syndecan family, was discovered and sequenced in the late 90s (Mali et al. 1990). Its description was soon followed by that of the three other members of the family. Virtually, all epithelial tissues express syndecan-1, typically located at the basolateral surface. Syndecan-1 has been detected in cells of the skin, oral cavity, lips, tonsils, salivary glands; in the epithelium of the gastrointestinal system including the esophagus, stomach, intestine, gall bladder, pancreatic duct cells, and hepatocytes; in the urogenital system including the kidney, urinary bladder, prostate, seminal vesicle, epididymis; in the respiratory system including the lungs and bronchi; and in the reproductive organs of females including the breasts, uterus, and placenta (Kind et al. 2019). The members of the syndecan family are phylogenetically highly conserved transmembrane proteoglycans, consisting of extracellular (EC), transmembrane (TM), and intracellular cytoplasmic (CD) domains. Meanwhile, the constant C1 and C2 regions of the intracellular domains show a high degree of homology among the family members, the extracellular domains display considerable heterogeneity.

The extracellular domains of syndecan-1 and syndecan-3 carry both heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycan (GAG) side chains (Fig. 3.1), while syndecan-2 and -4 are exclusively glycanated by HS chains. These negatively charged GAG chains mediate most of the syndecan-1 interactions with a

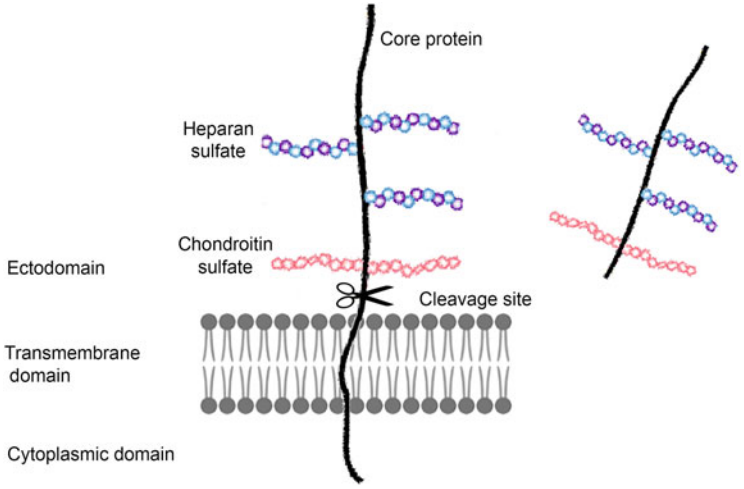

Fig. 3.1 Syndecan-1 is a transmebrane proteoglycan. According the protein is built up by intracellular, transmembrane, and extracellular domains. The latter carries two chondroitin sulfate and three heparan sulfate chains. In consequence of the negative charge of the sugar chains, syndecan-1 can establish interactions with several, basic molecules participating this way in cell regulation. The extracellular domain has more cleavage sites for proteases resulting the liberation of the EC, called shedding, that provide additional tasks for this domain. Intracellular domain, internalization of the whole molecule, or the shedded domain can exert further effect in the cytoplasm or in the cell nuclei

plethora of basic molecules. Syndecans may bind growth factors and cytokines located in the extracellular matrix (ECM), as well as cell surface molecules such as integrins. So far, around a hundred interacting partners have been identified and this number is ever-increasing (Stepp et al. 2015). In many of these interactions, syndecan-1 acts as a ternary receptor, facilitating the signaling of growth factors through their specific receptors, this way supporting the outside-in signaling from the microenvironment. To communicate with the microenvironment, besides its GAG chains, syndecan-1 also utilizes its EC domain. The cytoplasmic domain establishes interactions via its constant and variable regions, associating with cortactin, tubulin, and signaling molecules such as CASK, Fyn, Src, NF1, syntenin, synbindin, α6β4, etc. The four syndecan-family members also interact with each other, thereby creating an enormous diversity and complexity to their functions (Roper et al. 2012; Kleiser and Nyström 2020; Gondelaud and Ricard-Blum 2019; Wang et al. 2014a).

3.2 Different Locations, Different Partners, Different Functions

According to the classical description, syndecan-1 resides on the cell surface as a transmembrane proteoglycan, but it can also be found in the nuclear compartment, the cytoplasm, and the ECM, and as a soluble shed biomolecule in body fluids. Moreover, recent studies reveal that its functions are strictly related to its localization.

One of the best-known characteristics of syndecan-1 is the **shedding** of its EC domain from the cell surface. Although this, as a part of protein renewal, is a physiological process (Subramanian et al. 1997; Jalkanen et al. 1987), several pathological events go together with increased shedding. Enzymes participating in shedding include matrix metalloproteinases (MMPs), and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), thrombin, plasmin, all of them having individual cleavage sites (Bertrand and Bollmann 2019). Besides the action of these proteases, heparanase (Rangarajan et al. 2020), the only mammalian heparin liase enzyme, also supports the shedding of syndecan-1, in part by shortening its GAG chains, and by stimulating the production of MMP9 through the ERK pathway (Purushothaman et al. 2008). Signaling molecules attached to the shed syndecan-1 are also removed from the cell surface, which suppresses their actions on the cells, while the factors dwell in the ECM. Increased amount of circulating syndecan-1 is an active participant of several pathological events, including inflammation (Gao et al. 2015), sepsis (Ostrowski et al. 2013), coagulopathies and pulmonary embolism (Lehnert et al. 2017) cardiomyopathy (Vanhoutte et al. 2007a), lung diseases (Li et al. 2019), and tumor invasion (Szarvas et al. 2018b).

The presence and the role of heparan sulfate in the **cell nucleus** has been debated when in the 80s, Ishihara and his coworkers published their results, demonstrating

the presence of heparan sulfate in the nucleus of hepatocytes (Ishihara et al. 1986; Fedarko and Conrad 1986). Their results did not receive appreciation for a long time; the first supporting publications were published at the turn of the twenty-first century. It has been shown that heparan sulfate, isolated from human livers inhibits the activity of nuclear topoisomerase I, in cooperation with bFGF (Kovalszky et al. 1998). Later, with the advent of confocal microscopy, the entire syndecan-1 molecule has been shown to translocate to the nucleus in a tubulin-mediated manner (Brockstedt et al. 2002). As HS chains of syndecan-1 bind bFGF (Filla et al. 1998), the growth factor also coprecipitates with syndecan-1 in the nuclear extract. Nuclear heparan sulfate and bFGF facilitate each other's nuclear uptake (Hsia et al. 2003). The localization of syndecan-1 in the nucleus is a characteristic feature of tumor cells. This implies that it is in very low representation in the nuclei of normal cells. Nuclear HS interferes with the action of topoisomerase I and II (Kovalszky et al. 1998), as well as AP1, and Ets1 (Dudás et al. 2000; Hollósi et al. 2020). Shed syndecan-1, lacking the transmembrane and cytoplasmic domains but with preserved HS chains has also been detected in the nucleus, indicating that this proteoglycan can internalize and translocate there, promoting histone acetylation (Stewart et al. 2015). Next, it was discovered that the nuclear syndecan-1 level is regulated by heparanase, and HS chains most probably interfere with the transcriptional activity of genes in tumor cells (Purushothaman et al. 2011a). The presence of heparanase in the nucleus results in fast downregulation of nuclear syndecan-1, and consecutive enhancement of histone acetyltransferase (HAT), known to be inhibited by HS (Purushothaman et al. 2011b). This mechanism assigns an aggressive phenotype in myelomas (Purushothaman et al. 2011b). Nuclear localization was detected in malignant mesothelioma, adenocarcinoma of the breast, lung cancer, hepatocellular carcinoma, and neuroblastoma (Zong et al. 2009; Kovalszky et al. 1998; Chen and Sanderson 2009; Stewart et al. 2015).

The functions of syndecan-1 related to the nucleus were separated from the functions related to the cell surface by transfecting a fibrosarcoma cell line with various syndecan-1 constructs, one with a preserved and another with a deleted RMKKK nuclear localization signal. Nuclear translocation of syndecan-1 hampered the proliferation of fibrosarcoma cells compared to the mutant lacking the nuclear localization signal. The growth inhibitory effect of nuclear syndecan-1 was accompanied by a significant accumulation of cells in the G0/G1 phase, which indicated a possible G1/S phase arrest. By combining multiple, unsupervised global transcriptome and proteome profiling approaches with functional assays, the molecular mechanisms that governed nuclear translocation and its related functions could be followed. The transforming growth factor β (TGF-β) pathway was activated by nuclear syndecan-1, and expression levels of three genes were significantly altered with the deletion of the nuclear localization signal: early growth response 1 (EGR-1), never-in-mitosis gene a-related kinase 11 (NEK11), and dedicator of cytokinesis 8 (DOCK8). Moreover, these genes were coupled to growth and cell-cycle regulation. Nuclear translocation of syndecan-1 influenced the activity of several other transcription factors including E2F, NFκβ, and OCT-1 (Szatmári et al. 2017). Mapping the nuclear syndecan-1 interactome by proteomic analysis after

coimmunoprecipitation in a malignant mesothelioma cell line identified many interacting proteins, and pathways related to cell proliferation, RNA synthesis, splicing, and transport (Kumar-Singh et al. 2020). Furthermore, loss-and gain-of-function experiments showed that syndecan-1 influences RNA levels in mesothelioma cells. The results identify a proteomic map of syndecan-1 nuclear interactors in a mesothelioma cell line and suggest a previously unknown role for syndecan-1 in RNA biogenesis.

Nuclear syndecan-1 also regulates the epithelial–mesenchymal plasticity in tumor cells. Loss of nuclear syndecan-1 associated with cellular elongation and an E-cadherin-to-N-cadherin switch during TGF-β1-induced epithelial–mesenchymal transition (EMT) in human lung adenocarcinoma cells (Kumar-Singh et al. 2021), and nuclear translocation of syndecan-1 contributed to the repression of mesenchymal and invasive properties of human B6FS fibrosarcoma cells.

Increasing number of publications call attention to the potential **of syndecan-1 in the regulation of intermediary metabolism**. In the liver, syndecan-1 functions as a cell surface receptor of lipoproteins on hepatocytes, regulating the uptake of remnant lipoproteins (Stanford et al. 2009; Wilsie et al. 2006). It also functions as a triglyceride receptor (Foley et al. 2013), and it is involved in their intracellular clearance (Reszegi et al. 2021b). Overexpression of syndecan-1 downregulates the amount of fatty acid synthase (FASN), one of the most critical player of lipid synthesis in the liver, and protects against de novo lipid production (Reszegi et al. 2021b). This raises the possibility that syndecan-1 can interfere with metabolic events taking place in the cytoplasm, such as glucose and lipid metabolism or mitochondrial functions. Experimental evidence indicates that syndecans are also implicated in the whole-body energy metabolism (De Luca et al. 2010).

The effect of syndecan-1 also depends on the cell types that carry the proteoglycan. The majority of epithelial cells express syndecan-1 on their cell surface. The functions and cellular interactions described above can be detected on all epithelial cells, but differences can also be seen. For example, syndecan-1 as a remnant lipid receptor is characteristic for hepatocytes (Stanford et al. 2009). By recruiting and binding inflammatory cytokines, shed syndecan-1 creates a cytokine gradient. As a result of its interaction with E- and P-selectin on the endothelium of blood vessels, syndecan-1 hinders the rolling of leukocytes and their subsequent extravasation (Voyvodic et al. 2014). Experiments carried out on SDC-$1^{-/-}$ mice revealed that the actions described above make syndecan-1 essential in limiting lung inflammation (Brauer et al. 2016) (Gopal 2020). Furthermore, besides attenuating inflammation, syndecan-1 protects against apoptosis of bronchial epithelial cells by facilitating Met signaling (Brauer et al. 2016). Circulating exosomes were also found to carry syndecan-1 cargo. Delivery of these exosomes into the lung attenuates the lipopolysaccharide (LPS)-induced inflammation and decreases edema via acting on pulmonary microvascular endothelial cells (Gopal 2020).

Syndecan-1 also supports the optimal resolution of myocardial infarction. It decreases the inflammatory reaction at the injured area, and attenuates the activity of MMPs, in this way protecting against type I collagen degradation, and the adverse infarct healing with concomitant cardiac dilatation (Vanhoutte et al. 2007b).

Presence of syndecan-1 regulates the motility of differentiated M2 macrophages, promotes the clearance of the inflammatory cells, and acts against the development of arteriosclerotic plaques (Voyvodic et al. 2014; Angsana et al. 2015). In the liver, shed syndecan-1 also protects against the development of fibrosis, by removing TGFβ1 and thrombospondin from the vicinity of the myofibroblasts (Regős et al. 2018).

HS chains of syndecan-1 can promote the uptake of viruses. One of the best-known examples is that of the hepatitis C virus (HCV) (Grigorov et al. 2017; Zhang et al. 2017). The virus binds to the HS chains of syndecan-1 and subsequently interacts with tetraspanin (CD81), which indicates the cooperation of the two molecules in the virus uptake. The virus in the infected hepatocytes further enhances the expression of syndecan-1, resulting in enhanced uptake of LDL and VLDL. Although HCV is capable of utilizing the HS chains of other proteoglycans as well, syndecan-1 as the major proteoglycan of hepatocytes is the most potential candidate to promote HCV entry (Shi et al. 2013). Human papilloma virus (HPV) 16 and 18 dwelling in the ECM on heparanase cleaved HS chains of syndecan-1 efficiently infect new epithelial cells of the uterine cervix (Surviladze et al. 2015). Besides CD4, syndecan-1 also acts as an attachment receptor of human immunodeficiency virus (HIV) on macrophages (Saphire et al. 2001).

3.3 Syndecan-1 and Signal Transduction

HS chains of syndecan-1 can bind growth factors such as bFGF2, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), as well as cytokines, interleukins, etc. These connections facilitate the interactions of growth factors with their cell surface receptors, initiating outside-in signaling. The best-known examples for these mechanisms are receptor tyrosine kinases, including EGF, cMet, FGF, PDGF VEGF receptors, utilizing the RAS-Raf-Erk, and the RAS-PIK3C-Akt-mTor pathways (Montor et al. 2018). The ligand–receptor interactions initiate signaling cascades regulating a plethora of cellular functions (Gross and Rotwein 2017). Besides the tyrosine kinase receptors, syndecan-1 is also involved in the Wnt signaling (Ren et al. 2018; Pataki et al. 2015). Furthermore, syndecan-1 and -4 with the cooperation of protein kinase Cα (PKCα) decreases calcium influx and cytosolic calcium level, by inhibition of TRCP calcium channels, a mechanism that regulates cell adhesion (Gopal et al. 2015). The effect of syndecan-1 is tissue specific and it depends on the variety of ligands and receptors present in a particular cell type. Thus, syndecan-1 orchestrates and regulates a plethora of interactions both in physiological and pathological conditions.

3.4 Syndecan-1 and Cancer; Does it Protect or Promote?

Soon after the cloning of human syndecan-1, Jalkanen and coworkers discovered that syndecan-1 inhibits the malignant transformation of human keratinocytes (Inki et al. 1994b). Since that time several experimental and clinical studies were carried out to find the mechanisms whereby syndecan-1 may inhibit or promote the development and progression of various malignant tumors. As syndecan-1 is a proteoglycan, the implication of GAG chains became one of the first potential candidates for this task. In contrast with the protein core, GAG chains and in particular HS chains, which are further modified by sulfation have a high variability (Sanderson et al. 1994; Dennissen et al. 2002; Mundhenke et al. 2002). The sulfated and acetylated regions form separated domain structures further supporting the versatility of the sugar chains (Lindahl and Li 2009). Analysis of GAG–protein interactions revealed that several proteins require well-defined HS structures for binding. Thus, different HS structures on the same core protein differently affect the actual function of the molecule (Kjellén and Lindahl 2018). This implies that syndecan-1 influences not only the behavior of the tumor cells, but also the cells in the tumor stroma, and the outcome depends on the tumor type and cellular localizations (Su et al. 2007; Ahmed Haji Omar et al. 2013; Handra-Luca 2020). Aberrant localization of syndecan-1 in the tumor cells also influences their behavior. Although the mechanism of syndecan-1 retention in the cytoplasm is not clearly understood, it is generally related to worse outcome.

Enhancement of syndecan-1 shedding is a typical feature of cancers. Sheddases, participating in this activity, are the same as those working under normal conditions; however, they act in an unregulated fashion. They remove the functional extracellular domain of syndecan-1 from the cell surface. The regulatory factors retained on the HS chains will interact with the stromal cells, interfere with their signaling, facilitate tumor angiogenesis, and proliferation. Such matrix bound syndecan-1 is capable of trapping migrating tumor cells, this way supporting the establishment of a tumor cell niche (Rangarajan et al. 2020; Manon-Jensen et al. 2010). Shed syndecan-1 in the circulation is a potential indicator of chemotherapy resistance (Szarvas et al. 2018a). Epithelial–mesenchymal transition (EMT), an indispensable process for tumor invasion, has been associated with syndecan-1. In prostate carcinoma, Zeb1 transcription factor crucial for the EMT process binds to the promoter region of syndecan-1 and silences its expression. It seems that the EMT process related to syndecan-1 does not necessarily involve TGFβ1 and vice versa, the canonical TGFβ1-initiated EMT can also be developed without the involvement of syndecan-1 (Couchman 2021; Yang et al. 2020). Syndecan-1 can be detected in the circulation as a component of the extracellular microvesicles that can discriminate between high- and low-grade gliomas (Indira Chandran et al. 2019). The concentration of circulating syndecan-1 positive myeloma microparticles correlates with the tumor burden giving a chance for individual monitoring of multiple myeloma progression (Krishnan et al. 2016; Yang et al. 2020).

3.5 miR Regulation of Syndecan-1 Expression in Tumors

An increasing number of publications report that the function of syndecan-1 is regulated by microRNAs (miR) in various cancers. Their effects can be stimulatory or inhibitory, depending on both the types of tumors and the miRNA. Downregulation of syndecan-1 by liver-derived miR 122–5p enhances mammary cancer motility (Uen et al. 2018). miR 10a via binding to the syndecan-1 3′ untranslated region supports the proliferation and migration of squamous carcinoma cells in the skin (Xiong et al. 2020). Binding of the same miR in MDA-MB-231 mammary cancer promotes cell proliferation and migration capacities by upregulating Rho-kinase activity, concluding to cytoskeleton modulation and downregulation of E-cadherin (Ibrahim et al. 2012). In case of prostate cancer, syndecan-1 can facilitate the progression of the tumor into a more aggressive phenotype, by cooperating with miR126 and miR149 (Fujii et al. 2015a). On the contrary, downregulation of syndecan-1 by the same miR results in the differentiation of urothelial cancer (Fujii et al. 2015b). Increased expression of miR302 inhibits ovarian cancer cell proliferation by targeting syndecan-1 (Guo et al. 2015). Overexpression of syndecan-1 in pancreatic cancer is related to poor prognosis and EMT. Its downregulation by miR494 inhibited tumor progression. It seems that not only miRs regulate syndecan-1 expression, but it works the other way around, as well. Syndecan-1 is able to influence the selection of exosome's miR cargo, secreted by lung cancer cells. Furthermore, depletion of syndecan-1 facilitates the production of oncogenic miR (Parimon et al. 2018). MiR-331-3p miRNA maturation, mediated by syndecan-1, promotes EMT of prostate cancer (Fujii et al. 2016).

3.6 Syndecan-1 Is a Differentiation Marker with a Prognostic Value in Solid Tumors

Syndecan-1 expression correlates with tumor cell differentiation and prognosis in solid tumors. Its cell surface reactivity serves as a differentiation marker, and it is present in all epithelial malignancies, whereas its expression is lower in mesenchymal and hematological malignancies and in less differentiated tumor components (Fig. 3.2).

The normal syndecan-1 level and its cellular localization in a particular tissue are crucial to understand the sequential changes involved in malignant transformation, tumor progression, and tumor dissemination. Experimental overexpression of full-length syndecan-1 enhances cell–ECM cohesion and restricts cell migration (Zong et al. 2011), whereas the loss of the syndecan-1 ectodomain from the cell

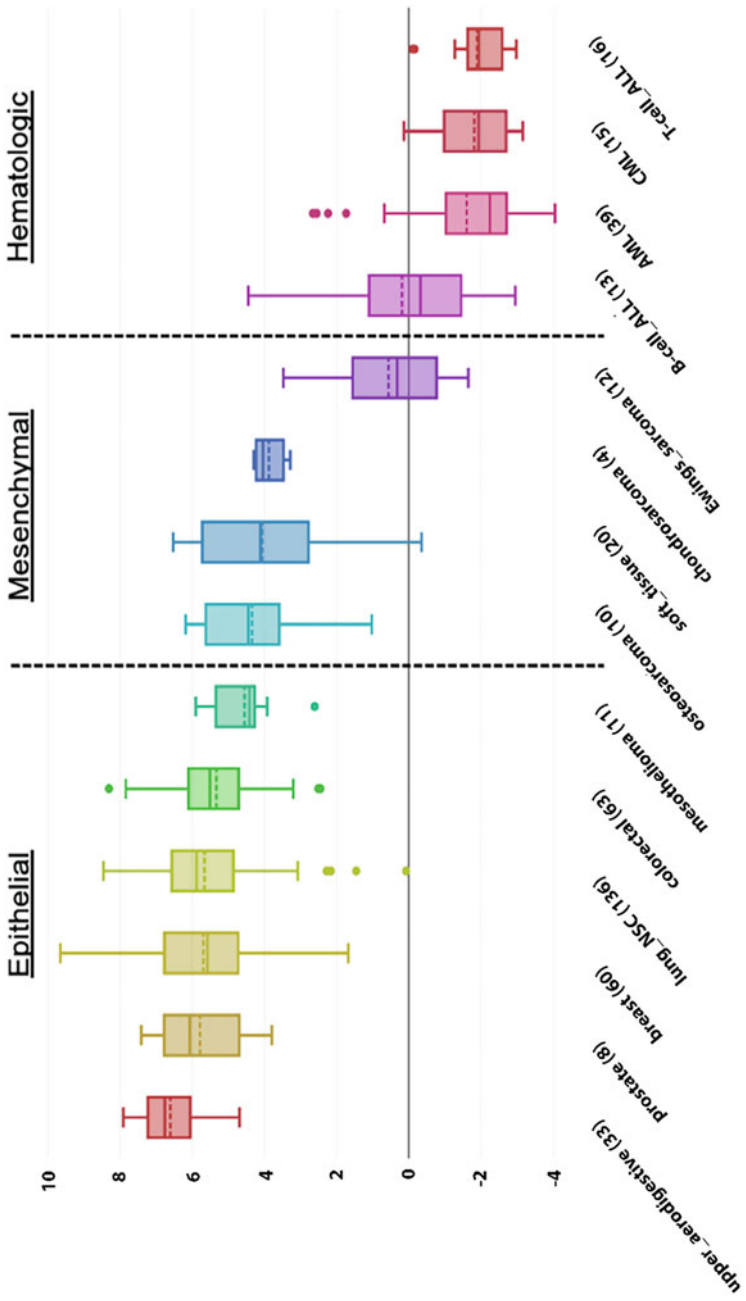

Fig. 3.2 Relative syndecan-1 mRNA expression in tumor cell lines. Data are obtained from the Broad Institute Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle). Numbers within parenthesis represent the number of analyzed cell lines within the same tumor type

surface increases the migratory capacity of tumor cells (Teng et al. 2012). Thus, cell bound, stromal, and shed syndecan-1 carry tissue-specific prognostic information in individual tumor types. The tremendous body of literature related to the involvement of syndecan-1 in various cancers is seemingly contradictory but considering the localization of syndecan-1, in addition to its expression level might lead to a better understanding of the complex regulatory mechanisms and related prognostic significance in each tumor type.

The expression of **cell surface syndecan-1** in tumor tissue is context-specific. For instance, compared to normal epithelial cells, decreased syndecan-1 expression has been found during malignant transformation, and reduced cell membrane syndecan-1 immunoreactivity was observed in many epithelial malignancies connected to various stages of tumor progression.

Syndecan-1 present in **the stromal component** of different malignant tumors generally indicates poor prognosis through the promotion of tumor cell invasion, dedifferentiation, and development of metastasis (Mukunyadzi et al. 2003; Ito et al. 2003). A shift of syndecan-1 from epithelial to stromal cells was observed in various solid tumors during their progression (Mennerich et al. 2004), and a growth promoting loop could be observed between breast cancer cells and its stoma (Maeda et al. 2004), where induction of syndecan-1 expression in stromal fibroblasts stimulated the growth of breast cancer cells. High level of shed **soluble syndecan-1 is** generally associates with poor prognosis and correlates to tumor burden, cancer invasiveness, and the risk for metastasis (Yang et al. 2007; Su et al. 2007; Szarvas et al. 2018a; Kurokawa et al. 2006; Malek-Hosseini et al. 2017).

The effects of syndecan-1 in malignant tumors depend on different factors. Of major importance is the localization; whether it is present in the tumor cell membrane or in the nucleus or extracellularly in the tumor stroma. The tumor cell phenotype and tissue from which the tumor originates are other such factors.

Thus, in **squamous carcinoma** of the head neck and larynx, decreased cell surface syndecan-1 expression in epithelial cells is associated with tumor aggressiveness and poor prognosis (Kurokawa et al. 2006; Inki et al. 1994a). In nasopharyngeal carcinoma, it correlates with advanced clinical stages and poor outcomes (Pulkkinen et al. 1997). Soluble syndecan-1 levels reflect the tumor burden, and decreased serum levels of syndecan-1 is predictive for favorable outcome (Anttonen et al. 2006). In squamous oral and cutaneous carcinoma, stromal syndecan-1 was inversely correlated with tumor grade and invasiveness (Ahmed Haji Omar et al. 2013; Máthé et al. 2006).

The presence of syndecan-1 is associated with favorable outcome in both **lung cancer** (Anttonen et al. 2001) and pleural **malignant mesothelioma** (Kumar-Singh et al. 1998). Generally, adenocarcinomas show higher cell surface and soluble syndecan-1 levels than primary malignant mesotheliomas of the pleura. Considering this feature, syndecan-1 has been proposed as a diagnostic marker in distinguishing mesotheliomas from metastatic adenocarcinomas (Gulyás and Hjerpe 2003; Saqi et al. 2005; Mundt et al. 2014). In squamous cell lung carcinoma, low syndecan-1

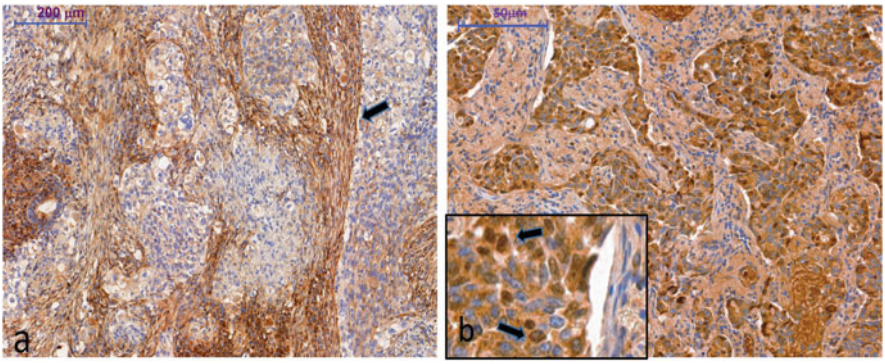

Fig. 3.3 Examples for the aberrant expression of syndecan-1 in lung adenocarcinomas. a. Vanish of syndecan-1 from the cell surface of the tumor cell parallel with its upregulation in the tumor stroma. Original magnification 10×. b. Loss of syndecan-1 from the surface of the tumor cells, and aberrant overexpression in all other compartment of the lung adenocarcinoma, showing intranuclear, cytoplasmic, and stromal positivity. Original magnification 20 ×

expression is associated with unfavorable outcome, and the majority of NSCLC express cell surface syndecan-1 reactivity as demonstrated by immunohistochemistry of tumor tissue, and elevated syndecan-1 levels in the serum associates with poor outcome in both NSCLC and SCLC (Linnerth et al. 2005; Joensuu et al. 2002; Anttonen et al. 2003) (Fig. 3.3).

The expression of syndecan-1 is induced in the stroma of **gastric cancer**, where its presence has been correlated with poor prognosis, and its immunohistochemical demonstration has been suggested as a potentially prognostic factor in patients with stage I gastric cancer (Wiksten et al. 2000). When syndecan-1 was present in the epithelial tumor cells, this correlated to a longer survival (Hu et al. 2014), whereas stromal syndecan-1 expression correlated with shorter survival (Wiksten et al. 2001). Low expression of syndecan-1 is significantly correlated with the invasion and metastasis of gastric carcinoma (Chu et al. 2008).

In the human **liver,** syndecan-1 is the major proteoglycan (Roskams et al. 1995) and its expression sometimes becomes intracellular in primary liver tumors (Roskams et al. 1998). As the majority of liver cancers develop in cirrhotic livers, characterized by elevated syndecan-1 expression, **hepatocellular carcinomas** developed on cirrhotic base express ample amounts of syndecan-1 (Regős et al. 2020). In hepatitis C-derived cancer, this increase is significant. In contrast, the loss of syndecan-1 expression is a typical feature of hepatocellular carcinomas without cirrhosis, when its expression is also reduced at the mRNA level. Both kinds of cancers have high metastatic potential and poor prognosis (Matsumoto et al. 1997). Although elevated or retained cell surface syndecan-1 indicates better prognosis, both tumors are characterized by increased proteoglycan shedding, which significantly associates with poor survival (Nault et al. 2013). Overexpression of syndecan-1 in the liver of transgenic mice exerts a protective role in a diethylnitrosamine-induced mouse hepatocellular cancer model. Compared to the wild-type

mice, their tumors occurred with 6-month delay, and in spite of the enhanced syndecan-1 shedding, they retained cell surface syndecan both on hepatocytes and tumor cells (Reszegi et al. 2021a).

Another interesting question is whether the liver tissue reacts with altered syndecan-1 expression to the presence of metastatic tumors? Liver is a primary destination of colon cancers. According to the authors' personal observation, syndecan-1 expression increased in the peritumoral area, the significance of this, however, needs further evaluation (Regős et al. 2020). Syndecan-1 expression is heterogeneous in **intrahepatic cholangiocarcinoma**. High level of syndecan-1 is associated with longer survival, whereas reduced expression of syndecan-1 is correlated with lymph node metastasis and poor prognosis (Harada et al. 2003).

In **pancreatic cancer**, increased levels of membrane syndecan-1 reactivity have been found (Conejo et al. 2000). In this tumor, epithelial syndecan-1 expression predicts better prognosis in the resectable disease, whereas stromal syndecan-1 expression is an independent adverse prognostic factor (Juuti et al. 2005).

Loss of epithelial syndecan-1 is associated with advanced clinical stage and poor prognosis in **colorectal cancer** (Lundin et al. 2005). Selective expression of syndecan-1 in tumor-initiating cell lines suggests a role for syndecan-1 in cancer stem cells (Suhovskih et al. 2015). Syndecan-1 shedding is increased in colorectal cancer and it renders tumor cell's resistance to chemotherapy (Wang et al. 2014b). Moreover, different research groups found either tumor promoting or tumor inhibiting effects in colorectal cancers (Kim et al. 2015; Hashimoto et al. 2008).

The functions of syndecan-1 in **mammary cancer** depend on the location of the proteoglycan, the extent of its shedding, and the subtypes of the cancer itself (Nikolova et al. 2009). The majority of the reports emphasize that the tumor cells express increased amount of syndecan-1 on their cell surface. Its expression is also induced in the stromal cells adjacent to the cancer, particularly in tumors exhibiting an aggressive phenotype (Lendorf et al. 2011). In another publication, the loss of epithelial syndecan-1 correlated with the syndecan-1 stromal expression and was found to be significant as a poor prognostic factor (Loussouarn et al. 2008). The final conclusion of a recent meta-analysis of 1305 mammary cancer cases corroborated the general opinion that high expression of the proteoglycan indicates poor prognosis (Qiao et al. 2019), regardless if it occurs on the cell membrane, in the cytoplasm, or in the tumor stroma. Studies from an in vitro breast cancer model have also suggested that syndecan-1 directly participates in tumor cell spreading and adhesion. In a breast cancer cell line, overexpression of wild-type syndecan-1 increased cell proliferation, whereas overexpression of constitutively shed syndecan-1 influenced the migratory capacity of the cells (Nikolova et al. 2009).

Syndecan-1 expression is induced in the stroma of invasive breast carcinomas (Stanley et al. 1999), and studies link the worse prognosis of breast carcinoma patients with stromal syndecan-1 expression. Furthermore, epithelial syndecan-1 expression associates with negative ER status, whereas stromal syndecan-1 expression was associated with positive ER status (Leivonen et al. 2004). A recent study showed that the proportion of syndecan-1 positive cells correlated with tumor grade better than the amounts demonstrated by immunohistochemistry, nuclear grade, or

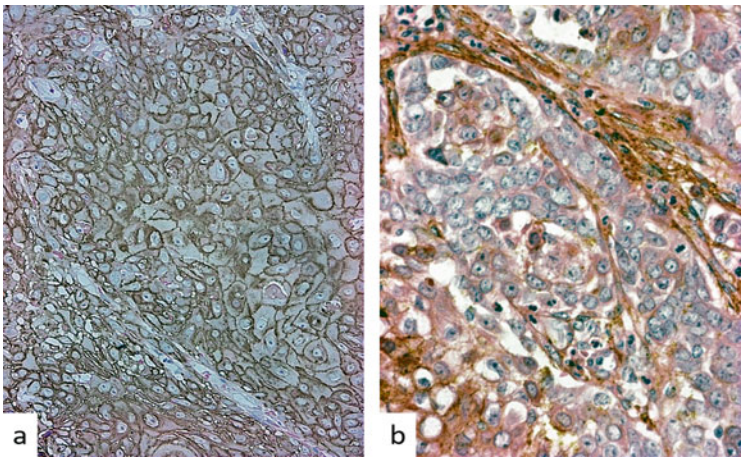

Fig. 3.4 Syndecan-1 immunostaining of squamous cancer of cervix. a. Intensive cell surface positivity of the tumor cells. Original magnification 20×. b. Loss of syndecan positivity from the tumor cell surface, together with immunopositivity of the fibroblasts in the tumor stroma. Original magnification 40 ×

localization of syndecan-1. The estrogen- and progesterone receptors both correlated negatively with syndecan-1 staining (Tiemann et al. 2014).

Syndecan-1 expression has a prognostic value also in **urothelial cancer**. Surface expression of syndecan-1 was inversely correlated with tumor stage in primary non-muscle-invasive bladder cancer (Kim and Park 2014), while high stromal syndecan-1 was associated with poor prognosis (Hatina et al. 2015; Szarvas et al. 2014).

In normal cervical squamous epithelium, cells with squamous metaplasia and koilocytotic atypia are positive for syndecan-1. During the progression of cervical intraepithelial lesions from low grade (LSIL) to high grade (HSIL), syndecan-1 expression successively diminishes. In **cervical squamous cell carcinomas (SCCs)**, syndecan-1 expression correlates with histological differentiation, being absent from most poorly differentiated tumors. Thus, loss of syndecan-1 seems to be an early event during cervical carcinogenesis and shows a clear association with histological differentiation grade (Inki et al. 1994c; Rintala et al. 1999). The progression of HSIL to early invasive squamous carcinoma and invasion into the lympho-vascular space associated with reduced syndecan-1 and HS–GAG expression along with lower overall survival rates compared to patients exhibiting high HS–GAG expression (Shinyo et al. 2005). Retained syndecan-1 expression on the tumor cell surface is a significantly positive prognostic marker, whereas its expression on tumor-associated fibroblasts indicates poor prognosis (Karászi et al. 2020) (Fig. 3.4).

Loss of epithelial syndecan-1 expression and induction of stromal syndecan-1 expression are associated with reduced survival in patients with **endometrial cancer** (Hasengaowa et al. 2005). In a xenograft model, upregulation of syndecan-1 developed proliferative and invasive/metastatic phenotypes. The growth advantage

conferred by syndecan-1 overexpression was accompanied by increased tumor angiogenesis (Oh et al. 2010). Increased stromal syndecan-1 staining was similarly a poor prognostic factor in **ovarian cancer** (Davies et al. 2004).

In normal prostate tissue, syndecan-1 is expressed mainly by the epithelial cells, while in tumors, an overall increase of syndecan-1 expression is observed in the tumor stroma, along with its disappearance from tumor epithelial cells (Suhovskih et al. 2013). In **prostate cancer**, intracytoplasmic accumulation of the proteoglycan in the tumor cells indicates aggressive behavior together with the early recurrence of the tumor (Kind et al. 2020). Furthermore, syndecan-1 level inversely correlates with tumor grade (Poblete et al. 2014). It has also been suggested that syndecan-1 may promote the regression of prostate cancer by stabilizing tumor initiating cells in experimental models (Shimada et al. 2013).

In mesenchymal tumors such as **malignant mesothelioma**, the expression of syndecan-1 correlates with epithelioid morphology and favorable prognosis. The underlying molecular mechanisms are complex and involve several critical genes and pathways. By studying the effect of syndecan-1 overexpression and silencing**,** the responses to modulated syndecan-1 expression were monitored by microarray (Szatmári et al. 2012) and proteomic analyses**.** Syndecan-1 overexpression had profound effects on several genes involved in the regulation of cell growth, cell-cycle progression, adhesion, migration, and ECM organization. Particularly, the expression of growth factors, interleukins, enzymes important for establishing the heparan sulfate sulfation pattern, ECM proteins, and proteoglycans were significantly altered. Syndecan-1 silencing had less powerful effect on the transcriptome compared to overexpression, which can be explained by the already low initial syndecan-1 level of these cells. Fourteen genes showed response to both up- and downregulation of syndecan-1. The "cytokine–cytokine-receptor interaction," TGFβ, EGF, VEGF, and ERK/MAPK pathways were significantly affected in both experimental settings. Interestingly, conditioned medium from syndecan-1 overexpressing malignant mesothelioma cells also exerted a paracrine inhibitory effect on endothelial cells proliferation, migration, and tube formation (Javadi et al. 2021). Multiplex soluble biomarker analyses of pleural effusions revealed that shed syndecan-1 levels are significantly lower in malignant mesothelioma effusions compared to adenocarcinoma, but significantly higher than that of benign samples (Javadi et al. 2020). Additionally, shed syndecan-1 and VEGF have prognostic value in malignant mesothelioma patients. Overexpression of the full-length syndecan-1 enhances B6FS **fibrosarcoma** and **malignant mesothelioma** cell lines' adhesion and inhibits the proliferation and migration of these mesenchymal tumors. On the other hand, syndecan-1 enhanced the proliferation, migration, and metastatic potential of HT-1080 cells (Péterfia et al. 2012) in mouse. Expression of syndecan-1 was also found in **malignant glioma** cells (Watanabe et al. 2006), and it is highly overexpressed in **dedifferentiated liposarcoma** (Zaragosi et al. 2015).

3.7 Unpredicted Functions of Syndecan-1 in Tumors

Although the role of syndecan-1 in the development and progression of tumors is exhaustively investigated, hardly any information is available about its role in the tumor metabolism. Experimental data provide more and more evidence that intermediary metabolism supports the biological activities of cancer cells. Along with this process, tumor cells establish strict cooperation with the resident cells of the ECM (Lau and Vander Heiden 2020). The details of these interactions depend on the particular types of tumors. As the liver is one of the major organs of intermediary metabolism, hepatocarcinogenesis in syndecan-1 transgenic mice offered a good approach to study the question. Upregulation of syndecan-1 on the surface of hepatocytes inhibited insulin receptor activation, PIKC3 and mTOR signaling, remodeling of ribosome biogenesis, and activation of Foxo1 and p53 proteins already known to regulate the intermediary metabolism including glucose and lipid homeostasis (Turi et al. 2019; Pelletier et al. 2018; Iadevaia et al. 2014; Lee and Dong 2017; Tzivion et al. 2011; Liu et al. 2019). Foxo1 is regulated by 14–3-3-zeta, an adapter protein with oncogenic potential, which was also inhibited by syndecan-1 (Matta et al. 2012; Reszegi et al. 2021a). As the proteoglycan is a well-known lipoprotein receptor on the surface of hepatocytes, its overexpression most probably modulates lipoprotein uptake from the blood circulation (Stanford et al. 2009). Furthermore, overexpression of FASN, a critical component of aberrant lipid metabolism with oncogenic potential in the liver, dramatically decreased as a result of syndecan-1 upregulation (Hu et al. 2020). Syndecan-1 can succesfully inhibit FASN, a critical promoter of nonalcoholic steato-hepatitis, hindering this way the disease progression to hepatocellular cancer.

3.8 Conclusion

Taken together, syndecan-1 is present in most epithelial malignancies and it carries prognostic information. Its regulation and expression pattern in different malignancies are however complex. In some cancers, such as head and neck cancer, lung cancer, and malignant mesothelioma, it indicates favorable prognosis when anchored to the cell membrane, and in contrast, it associates with poor prognosis when recovered in shed or soluble form in body fluids. In breast carcinoma, both in the epithelium and in stroma, syndecan-1 indicates mainly poor prognosis. A comprehensive characterization of syndecan-1 in malignant tumors requires further studies that consider not only the expression level and cell surface reactivity but also its various localization as these constitute key features determining the downstream effects and clinical outcome.

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Chapter 4
Syndecan-2 Biology and Its Role in Colorectal Carcinoma

Eok-Soo Oh and John R. Couchman

Abstract The mammalian syndecan family of transmembrane proteoglycans comprises four members. Syndecan-2 is widely expressed, notably in mesenchymal tissues and has roles in cell adhesion, extracellular matrix assembly and its turnover, as well as concentrating ligands, such as growth factors, cytokines, and morphogens at the cell surface. Although signaling through syndecan-2 core protein is incompletely understood, research has indicated roles for this receptor in colorectal cancer. Its expression in transformed epithelia has been related to increased aggressiveness and invasive potential. At the same time, it has been shown that syndecan-2 is subject to epigenetic regulation at the level of promoter methylation. Hypermethylated SDC2 shows promise as a marker of the earliest stages of colorectal cancer and clinical trials have been established. The biology of syndecan-2 is therefore potentially relevant to the progression of this and other cancers, but further work is required to fully understand its molecular functions.

4.1 Introduction

There are four mammalian members of the syndecan family of transmembrane proteoglycans. Each can bear heparan sulfate chains on their ectodomains, while syndecans-1 and -3 can also carry chondroitin or dermatan sulfate chains (Fig. 4.1). The four syndecans are derived from a common ancestor, there being a single syndecan in invertebrates of the Bilateria (Chakravarti and Adams 2006). It appears that at the invertebrate/vertebrate boundary, two rounds of gene duplication occurred, one branch having slightly larger core proteins than the other.

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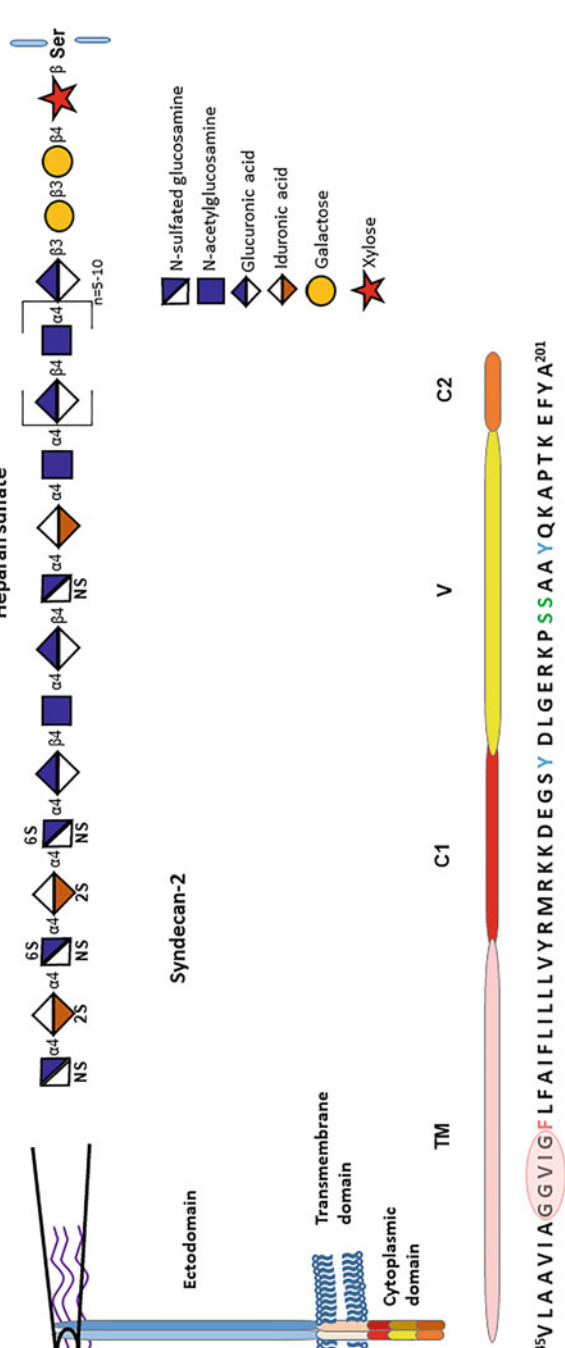

Fig. 4.1 Diagrammatic structure of syndecan 2 proteoglycan. Heparan sulfate chains are located toward the N-terminus, the composition of which is shown on the right. Below is shown the amino acid sequence of human syndecan-2 transmembrane (pink) and cytoplasmic domains. The GXXXG motif in the transmembrane domain that promotes dimerization is circled and the phenylalanine residue immediately adjacent to this motif is highlighted. This residue also contributes to dimer formation. The cytoplasmic domain has two conserved motifs, C1 and C2, either side of a variable (V) region specific to each syndecan, but nevertheless conserved across species. Tyrosine (blue) and serine (green) residues that are potentially phosphorylated are shown

Syndecan-2 is one of the smaller syndecans, along with syndecan-4 (Gopal et al. 2021). As part of having smaller core proteins, the cytoplasmic domains of these two syndecans are also smaller than that of the invertebrate ancestor as well as syndecans-1 and -3. Each of the four syndecans has a specific expression pattern, both in spatial and temporal terms (Gopal et al. 2021; Bernfield et al. 1999; Couchman 2010). Roles in neuronal guidance and stem cell regulation have been demonstrated in the invertebrate syndecans of *C. elegans* and *Drosophila* and similar trends can be seen in syndecan-3 of vertebrates (Rhiner et al. 2005; Johnson et al. 2004; Pisconti et al. 2016). However, although it is very likely that the four syndecans of mammals have embraced new roles, details are still incomplete.

Gene deletions of syndecans have been revealing. A common theme in syndecan-1 and -4 knockouts is that wound repair is delayed or impaired, though the mice are viable (Stepp et al. 2002; Echtermeyer et al. 2001). Indeed, double knockouts of syndecan-1 and -4 are also viable, but have alterations in, for example, epidermal cell morphology and junctions (Gopal et al. 2015). It has been noted several times that deletion of syndecans can lead to alterations in cadherin expression and function (Gopal et al. 2015; Kato et al. 1995; Gopal et al. 2017). Syndecan-3 knockouts have defects in hippocampus-dependent memory and enhanced long-term potentiation due to loss of interaction with heparin-binding growth-associated molecule (HB-GAM; Hienola et al. 2006). In skeletal muscle, loss of syndecan-3, which binds to Notch, leads to altered satellite cell interactions with their niche (Pisconti et al. 2010). To date, there has been a single report on syndecan-2 deletion in the mouse (Corti et al. 2019). There are defects in angiogenesis both in development and postnatal wound repair. The molecular basis appears to be that syndecan-2 forms a complex with $VEGFA_{165}$ (which is heparan sulfate-binding) and the receptor, VEGFR2. Moreover, it is proposed that the N-terminal 59 amino acids of the core protein provide information that results in enhanced 6-O-sulfation of the heparan sulfate chains, and which is required for the VEGF/VEGFR interactions. How core protein properties impact sulfation events in the Golgi apparatus is not yet understood. This region is distinct in syndecan-4 and does not enhance 6-O-sulfation, potentially explaining why it does not share the angiogenesis properties (Corti et al. 2019; Lambert et al. 2020). However, contrary data support the possibility that under some circumstances, syndecan-4 can promote angiogensis, pathogenic angiogenesis in particular (Echtermeyer et al. 2001; Pessolano et al. 2021; De Rossi et al. 2021). The syndecan-2 data are, however, consistent with angiogenesis deficits noted previously in zebrafish models using morpholinos directed to the proteoglycan core protein (Chen et al. 2004a).

4.2 Structure and Glycosylation

All four syndecans have cytoplasmic domains that can interact with the actin-based cytoskeleton and PDZ domain proteins that may regulate localization and trafficking (Gopal et al. 2021; Couchman 2010). The cytoplasmic domains of syndecans can be

resolved into three regions, two highly conserved, flanking a "variable" (V) region (Fig. 4.1). Each syndecan therefore has a distinct V region, likely enabling specific interactions with cytoplasmic signaling molecules, but details still remain sparse. The exception is syndecan-4, where interactions with α-actinin and protein kinase Cα have been demonstrated that can lead to enhanced cell-extracellular matrix (ECM) adhesion and the assembly of actin-based focal adhesion/contact organelles (Gopal et al. 2021; Couchman 2010; Bass and Humphries 2002; Elfenbein and Simons 2013; Mitsou et al. 2017). Syndecan-4 cytoplasmic domain is also the only one that has been structurally resolved. It forms twisted clamp dimers that are stabilized by interactions with specific inositol phospholipids (Lee et al. 1998). It is quite possible that syndecans-1, -2, and -3 cytoplasmic domains form similar structures. A tendency to form dimers is also seen with the single pass transmembrane domains, which can form dimers resistant to dissociating detergents such as sodium dodecyl sulfate (Choi et al. 2005). An NMR study of syndecan-2 transmembrane domain monomer has been reported (Li et al. 2019), showing a conventional helical structure. The inherent ability of syndecan transmembrane domains to form dimers is derived from a GXXXG sequence, also noted in glycophorin, for example (Choi et al. 2005; Teese and Langosch 2015; Dews and Mackenzie 2007). In the case of syndecan-2, this dimer formation is reinforced by a single phenylalanine residue immediately C-terminal of the GXXXG motif (Kwon et al. 2015).

In a very recent, illuminating study, it has now been shown that the ectodomains of all four syndecans are disordered and flexible. This may permit multiple interactions with other molecules on the cell surface, and adopt different conformations that facilitate complexes with other cell surface receptors (Gondelaud et al. 2021). This work was completed on core protein ectodomains lacking glycosylation, and it will be interesting to see in the future how glycosaminoglycan chains influence conformations. Moreover, given the transmembrane domain tendency to form dimers, it may be that their presence, along with resultant dimers may also constrain ectodomain conformation. This is already hinted at by the use of a syndecan-4 ectodomain dimer possessing a single cysteine bridge between two monomers (Gondelaud et al. 2021).

By virtue of the fact that heparan sulfate chains can interact with a myriad of proteins with many different functions, syndecans can be involved with a range of cellular processes. Included are many growth factors, cytokines, chemokines, enzymes, extracellular matrix macromolecules, and mediators of lipid metabolism. The structure and synthesis of heparan sulfate has been well documented (Lindahl et al. 2017; Xu and Esko 2014) and is summarized in Fig. 4.1. In essence, the chains are a series of repeating disaccharides, consisting of N-acetyl glucosamine and glucuronic acid. However, chains are extensively modified by sulfation and epimerization of the uronic acid residues to iduronate. A number of specific transferases combine to produce heparan sulfate chains of immense complexity and potential variability. Most heparan sulfate chains have domains of high sulfation interspersed with domains of low, or no, sulfation, with intermediate sulfation at the boundaries of these two domain types (Lindahl et al. 2017; Gallagher 2015). Despite this, it is apparent that heparan sulfate from a particular source, e.g., hepatocytes,

may have conserved overall characteristics (Gallagher 2015; Kjellén and Lindahl 2018). The extent of variability in chain length, and fine structure characteristics in vivo remains a challenging question.

4.3 Syndecan-2 Interactome and Signaling

As with all syndecans, syndecan-2 interactions on the cell surface or extracellular environment can involve both the glycosaminoglycan chains and regions of the core protein ectodomain (Gopal et al. 2021; Beauvais et al. 2009; Jung et al. 2019; Whiteford et al. 2011; Tsoyi et al. 2018). In the cortical cytoplasm, further interactions can involve the short cytoplasmic domain (Gopal et al. 2015, 2021; Couchman 2010; Bass and Humphries 2002; Elfenbein and Simons 2013). Since there are scores of potential ligands that can interact with heparan sulfate, derived from many families of proteins, e.g., cytokines, chemokines, enzymes, extracellular matrix macromolecules, dissecting specific signaling through syndecans has been a challenge. It is clear in some cases that syndecans can associate with other cell surface receptors, e.g., integrins, growth factor receptors, morphogen receptors, in turn regulating specific signaling pathways (Rhiner et al. 2005; Johnson et al. 2004; Bass and Humphries 2002; Jung et al. 2019; Whiteford et al. 2011; Tsoyi et al. 2018; Mundhenke et al. 2002; Bentzinger et al. 2013). Furthermore, the cytoplasmic domains of all syndecans are small and do not possess intrinsic kinase or phosphatase activities. Therefore, docking of cytoplasmic molecules to this domain is required for signaling (Gopal et al. 2021; Gopal et al. 2015). However, specific signaling has been demonstrated for syndecan-4, which involves the docking and activation of protein kinase Cα (Gopal et al. 2021; Mitsou et al. 2017; Oh et al. 1997; Keum et al. 2004). For syndecans-1 and -4, regulation of transient receptor potential canonical (TRPC) channels has also been demonstrated (Gopal et al. 2015; Mitsou et al. 2017). Alongside, all syndecans have been shown to interact with actin and tubulin cytoskeletal components, as well as junctional components and molecules involved in internalization and trafficking (Gopal et al. 2021; Gondelaud and Ricard-Blum 2019). Indeed, an important function of syndecans may be receptor/ligand internalization, with these processes being co-opted by a number of different pathogens. Examples include some viruses, bacteria, and malarial parasites (Gopal et al. 2021; Cagno et al. 2019; Teng et al. 2012; Ayres Pereira et al. 2016).

With respect to syndecan-2, there is still much to learn regarding specific signaling roles for the core protein. However, interacting proteins have been recorded and these have been curated in a comprehensive review (Gondelaud and Ricard-Blum 2019). Since then, there has been a study of the cardiac syndecan-2 interactome (Mathiesen et al. 2020). The data as a whole derive from affinity purification methods as well as interaction studies, so that in many cases the interactions could be direct or indirect.

Gondelaud and Ricard-Blum (2019) identify a core of 18 proteins that interact with all 4 mammalian syndecans. Of these, two are heparin/heparan sulfate-binding

(e.g., FGF2), while four are the syndecans themselves. This has important implications, since syndecans may therefore form hetercomplexes that render specific signaling pathways difficult to ascertain. Moreover, where specific affinity probes are used, for example, the cytoplasmic domain of a syndecan, complexation with endogenous syndecans may lead to copurification of interactors with ectodomains or heparan sulfate chains. Virtually all of the remaining 12 proteins that interact with all 4 syndecans do so by binding the conserved C1 and C2 regions of their cytoplasmic domains (Fig. 4.1). This is consonant with their highly conserved sequences (Bernfield et al. 1999; Couchman 2010) and supports the idea that syndecan-specific signaling may be engendered through the V regions that are distinct to each syndecan. Among these, 12 proteins are some that form submembranous networks, e.g., syntenin, two tyrosine kinases of the Src family, the cytoskeletal proteins cortactin and tubulin, a vesicle trafficking protein (TRAPPC4/synbindin), and one integrin subunit (β4; Gondelaud and Ricard-Blum 2019; Mathiesen et al. 2020). However, in the case of syndecan-2 that is largely mesenchymal in distribution, interactions with the predominantly epithelial α6β4 integrin may be infrequent.

Of the 56 interacting partners that are listed for syndecan-2, 25% are, somewhat surprisingly, lysosomal, while many others putatively bind to the heparan sulfate chains (Gondelaud and Ricard-Blum 2019). Only three proteins are listed as interacting with syndecan-2's cytoplasmic V region. These are caveolin-2, EphB2 and Sarm-1 (sterile α and toll interleukin receptor motif containing protein-1) which hydrolyses NAD^+ (Loring and Thompson 2020). The cardiac interactome study, using three complementary affinity-based techniques revealed remarkably few heparan sulfate-interacting partners, but did include three of the four cavins (Mathiesen et al. 2020). Alongside the interactions with caveolin-2 and lysosomal proteins, this suggests an important feature of syndecan-2 function relates to trafficking. Other interactors included G protein-related, ubiquitin-related, as well as cytoskeletal proteins (e.g., cortactin, which interacts with all syndecans).

The V region interaction with EphB2 adhesion protein raises an interesting further dimension, that of phosphorylation. This interaction requires phosphorylation of two tyrosine residues ($Y^{179}$and Y^{191}—human numbering), but not that of the C2 tyrosine (Y^{200}) (Fig. 4.1; Ethell et al. 2001). This promoted syndecan clustering and dendritic spine formation. Syndecan-2 phosphorylation also featured in a study of left-right asymmetry in *Xenopus* (Kramer et al. 2002). In this case, the two adjacent serine residues were phosphorylated by protein kinase Cγ ($S^{150/151}$—*Xenopus* numbering, corresponding to $S^{187/188}$ in human) (Fig. 4.1). Phosphorylation in right, but not left animal cap cells led to noncell autonomous signaling, perhaps suggestive of syndecan clustering once again.

A specific interaction of syndecan-2 ectodomain with the phosphatase CD148 (PTPRJ) is potentially important. This direct interaction was first identified in the context of integrin-mediated cell adhesion (Whiteford et al. 2011). The mechanism in fibroblasts involved both Src tyrosine kinase and the C2β isoform of PI3-kinase (Whiteford et al. 2011). Subsequently, the syndecan-2/CD148 axis has been found to regulate fibrosis (Tsoyi et al. 2018, 2021), and the depletion of CD148 signaling exacerbates experimental lung fibrosis (Tsoyi et al. 2021). These interesting data

recall previous work showing roles for syndecan-2 in extracellular matrix assembly (Klass et al. 2000; Galante and Schwarzbauer 2007; Jang et al. 2020). Moreover, syndecan-2 can mediate the internalization of TGF-βRI through a caveolin-mediated pathway (Shi et al. 2013), thereby moderating processes such as fibroblast activation and fibrosis. These properties are distinct to syndecan-2 and are not shared with its closest relative, syndecan-4. This may be advantageous in the event that syndecan-2 signaling in fibrosis becomes a potential target.

4.4 Syndecan-2 in Human Diseases

All cellular components of normal tissue are tightly regulated in response to external signals in order to perform appropriate and specific functions in tissue homeostasis. Since external stimuli vary in time and space, cells and tissues are in continual reciprocal interactions with their environment. One mechanism is to regulate the expression levels of cell surface receptors. Altered cell surface receptor expression in tissues either restores tissue homeostasis or induces changes that can become pathogenic. Syndecan-2 is a receptor now known to be aberrantly regulated in some diseases, notably cancers. When syndecan-2 was first characterized by cDNA cloning, it was named "fibroglycan," indicative of this syndecan family member having a strong bias toward mesenchymal cell expression (David et al. 1993). Indeed, it is commonly expressed in cells of mesenchymal origin, including fibroblasts, smooth muscle, endothelial, as well as neuronal cells, monocytes, and macrophages (Gopal et al. 2021; Bernfield et al. 1999; Ethell et al. 2001; Whiteford et al. 2007; Zhao et al. 2012; Noguer et al. 2009; Clasper et al. 1999; Essner et al. 2006). Therefore, acquired syndecan-2 expression in epithelial tissue that is normally low in expression creates new functions and changes tissue homeostasis, while decreased syndecan-2 expression in mesenchymal cells may also result in altered behavior.

A common trigger for altered syndecan-2 expression is inflammation, with chronic inflammation leading to diseases such as fibrosis. For instance, the inflammatory cytokines IL-1β, IL-6, and TGF-β1 upregulate syndecan-2 expression in myofibroblasts and also regulate the development of liver cirrhosis (Renga et al. 2014). However, increased expression of syndecan-2 in response to inflammation is not unique to this family member, but may be common across the family (Gopal et al. 2021; Couchman 2010; Teixeira and Götte 2020; Arokiasamy et al. 2020). For example, the expression of syndecan-2 along with other syndecan family members is also increased in myocytes after myocardial infarction (Wernly et al. 2019; Xie et al. 2012; Lunde et al. 2016), which may regulate cardiac ECM remodeling.

Syndecan-2 expression often changes in transformed cells. Overexpression of syndecan-2 is associated with the tumorigenic properties and poor prognosis of epithelial malignancies (Park et al. 2005; Mytilinaiou et al. 2013). Syndecan-2 promotes an aggressive phenotype of the MDA-MB-231 breast carcinoma cell line by combining with caveolin-2 and in part by regulating RhoGTPase (Lim and

Couchman 2014). In pancreatic cancer cells, when syndecan-2, through p120-GAP, activates Src in the absence of RACK1, mutated K-Ras activation induces cancer cell proliferation, spreading, and invasion (Betriu et al. 2021). Elevated syndecan-2 expression in skin-derived melanoma cells regulates their migratory properties (Lee et al. 2009).

Syndecan-2 expression is also increased in sarcoma progression. In the case of fibrosarcoma, a rare malignant tumor originating from fibroblasts, syndecan-2 regulates IGF-1-dependent actin cytoskeletal organization and cell motility (Mytilinaiou et al. 2017a) and TGFβ2-dependent fibrosarcoma cell functions such as cell adhesion (Mytilinaiou et al. 2013). In osteosarcoma, syndecan-2 plays a pro-apoptotic role and is therefore anti-oncogenic (Dieudonné et al. 2010). In addition, its increased expression in osteosarcoma cells was associated with reduced migration and invasion (Orosco et al. 2007). Since syndecan-2 generally has a pro-migratory function in carcinoma cells, it is possible to predict that its increased expression may have pro-oncogenic functions in these cells. Indeed, in colon cancer, one of the most studied carcinomas, the obtained syndecan-2 expression can influence a range of cancer cell behaviors. Syndecan-2 expression on the cell surface causes carcinoma cells to reorganize the cytoskeleton (Kusano et al. 2004), remodel the ECM (Jang et al. 2020), promote a more migratory and invasive cell phenotype or epithelial-to-mesenchymal transition (EMT) (Hua et al. 2020; Contreras et al. 2010), and angiogenesis (Noguer et al. 2009), all of which are all critical for carcinoma cells to establish primary tumors and secondary metastases.

4.5 Syndecan-2 in Colorectal Cancer

One of the best studied carcinomas related to roles of syndecan-2 is colorectal cancer. Several studies have shown that increased syndecan-2 is involved in the regulation of adhesion, proliferation, migration and invasion, cytoskeletal organization, and ECM assembly to regulate the tumorigenic activity of colon carcinoma (Klass et al. 2000; Han et al. 2004; Park et al. 2002).

As a cell surface receptor, syndecan-2 regulates the behavior of colon cancer cells in three different ways. First, as an adhesion receptor, syndecan-2 mediates intracellular signaling events in response to the external environment. Upon interaction with external ligands through the glycosaminoglycan (GAG) chains attached to the extracellular domain and/or the extracellular core protein itself, syndecan-2 triggers the transmission of signals from the extracellular environment into the cytosol to regulate the intracellular environment (Chen et al. 2004b; Mytilinaiou et al. 2017b), probably together with integrin-dependent signaling (Choi et al. 2009). The C-terminal EFYA sequence of syndecan-2 cytoplasmic domain interacts with syntenin-1, a scaffold protein containing two PDZ domains, which controls membrane localization and activation of Tiam-1, a guanine nucleotide exchange factor (GEF) of Rac (Grootjans et al. 1997; Lee et al. 2011; Choi et al. 2010). Syndecan-2 thus induces syntenin-1-Tiam1-mediated Rac activation, which leads to actin

filament assembly, for example, the production of filopodia, morphological changes, and increased cell motility (Choi et al. 2010). Syndecan-2 also regulates the expression and secretion of ECM components that have various functions in modulating cancer progression. For example, the engagement of the extracellular domain of syndecan-2 and the ECM transduces signals to activate PKCγ/FAK/ERK signaling pathway to induce the expression of MMP-7, which degrading a wide variety of ECM molecules, contributes to the remodeling of the matrix and favors colon cancer progression (Jang et al. 2016, 2017). Therefore, syndecan-2 expression regulates both intracellular and extracellular signaling events (Fig. 4.2).

Second, as a docking receptor, syndecan-2 regulates multiple extracellular events including receptor activation and the turnover of ECM. For instance, in the ECM, the binding of FGF-2 to syndecan-2 initiates a high local concentration of FGF-2 on the cell surface. This interaction facilitates efficient FGF-2 binding to the FGFRs with higher affinity than FGF-2 alone and induces FGF receptor activation which signals for cancer cell elongation (polarity) and motility (Clasper et al. 1999; Essner et al. 2006). Similarly, syndecan-2 increases the binding of $VEGF_{165}$ to VEGF receptor-2 (VEFGR2) and activation of VEGFR2 (Corti et al. 2019), which is necessary for angiogenesis in zebrafish development (Chen et al. 2004a). On the other hand, the binding of syndecan-2 to the pro-form of MMP-7 triggers the processing of pro-MMP-7 into active MMP-7 (Ryu et al. 2009), which results in activation of extracellular proteases. Activated MMP-7 may then enhance degradation of proteins of both ECM and cell surface (Gopal et al. 2015; Choi et al. 2012). As an ECM-degrading enzyme, activated MMP-7 degrades protein components such as collagen, fibronectin, and proteoglycans (Nagase and Woessner 1999; Miyazaki et al. 1990), as part of cell-induced ECM remodeling. Activated MMP-7 also cleaves cell adhesion receptors such as E-cadherin (Jang et al. 2016; McGuire et al. 2003), the major cell–cell adhesion receptor of epithelial adherens junctions (McCawley and Matrisian 2001). The resulting functional loss of E-cadherin in early-stage E-cadherin-expressing colon cancer cells may induce EMT with subsequently enhanced cell migration (Jang et al. 2016; Bates and Mercurio 2005). Similarly, MMP-7 mediates the cleavage of the extracellular domain of syndecan-2 which results in loss of syndecan-2 function at the cell surface, under conditions whereby cancer cell migration and invasion are increased (Choi et al. 2012, 2015).

Third, remodeling of the ECM by MMPs releases various bioactive fragments that further regulate numerous biological processes, including tumor growth and metastasis. Since syndecan-2 is itself cleaved by MMP-2, -7, and MT1-MMP, the shed proteoglycan is also involved in the colon cancer progression. First, shed syndecan-2 may not only release signal proteins engaged at the cell surface, but also increases their activity. FGF-2 bound to shed syndecan-2 interacts more efficiently with the FGF receptor than FGF alone. As a result, syndecan-2 can enhance angiogenesis via the FGFR signaling pathway (Clasper et al. 1999; Villena et al. 2003). In addition, shed syndecan-2 can bind to (unknown) receptors to regulate cell functions as ligands to impact gene expression. In fact, treatment with shed syndecan-2 directly increases the migration of HCT116 cells (Choi et al. 2015) and promotes capillary tube formation in microvascular endothelial cells of the brain

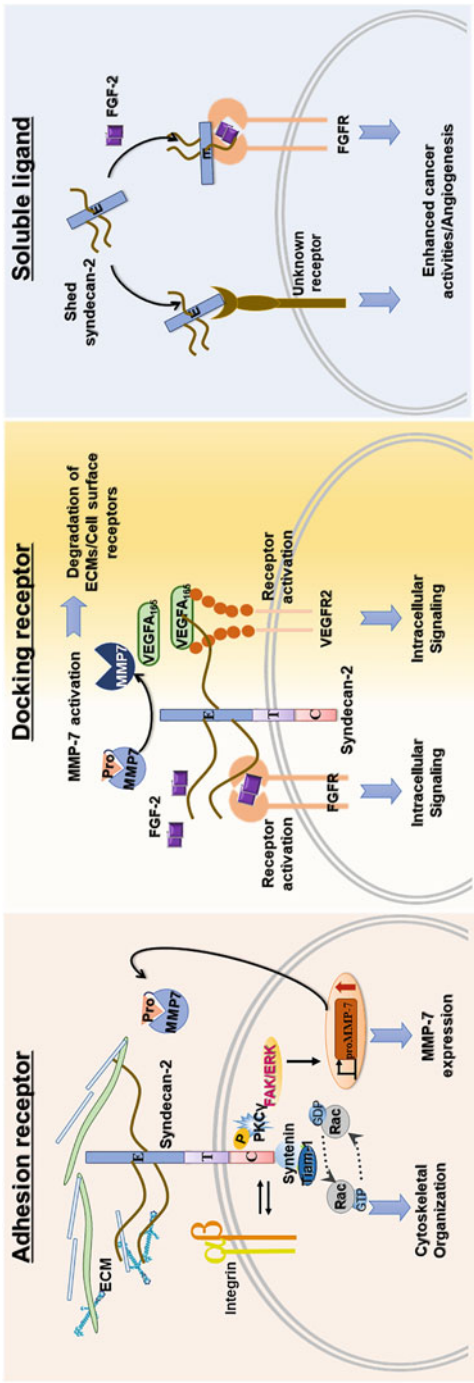

Fig. 4.2 The regulatory mechanisms of syndecan-2 in cancer. (**a**) In the context of an adhesion receptor, the extracellular domain of a syndecan-2 transduces signals from the extracellular environment to the cytosol, thereby regulating intracellular signaling events such as (i) cytoskeletal organization, and (ii) expression of ECM molecules including MMP-7. (**b**) As a docking receptor, a syndecan-2 can regulate extracellular events. When extracellular ligands bind to syndecan-2, (i) extracellular ligands such as growth factors bind with high affinities to their receptors and activate their receptor signaling and (ii) extracellular enzymes such as MMP-7 become activated. Activated MMP-7 cleaves ECM molecules and cell surface receptor such as syndecan-2, which produces shed syndecan-2 fragment in the ECM. (**c**) As a soluble ligand, (i) shed syndecan-2 bound FGF-2 activates FGF receptor signaling and (ii) shed syndecan-2 directly regulates various cancer activities including angiogenesis by unknown mechanisms

(Fears et al. 2006). Therefore, shed syndecan-2 can intensify angiogenic processes, thereby facilitating the growth and metastasis of cancer cells and promoting tumorigenic activities as a soluble ligand.

4.6 SDC2 Gene Methylation and Colon Cancer

In 2013, Oh et al. (Oh et al. 2013) published a study showing that SDC2 promoter hypermethylation could be detected in serum samples from colon cancer patients (i.e., cell free) with high specificity and sensitivity. Subsequently, several studies and literature surveys have supported this notion (Rasmussen et al. 2016; Rasmussen et al. 2017; Kim and Park 2018; Han et al. 2019; Su et al. 2021), with most research coming from South Korea and Denmark. There have been suggestions that SDC2 gene analysis can be used alongside other genes (e.g., TFPI2 or SEPT9; Zhang et al. 2021; Zhao et al. 2019). Promoter methylation can be common in early carcinogenesis, and since cancer cell exfoliation can occur before blood leakage into the colon, the hope is that potential markers such as this may detect the earliest manifestations of malignancy. Consistent with this, analysis has moved from serum to colon lavage fluid and stool DNA samples.

Two clinical trials are in progress (NCT03146520, NCT04304131) to assess the utility of stool-derived SDC2 methylation status as a basis for colorectal carcinoma detection (Kim et al. 2021). A test kit based on this premise has now been developed by Genomictree Inc., Daejon, S. Korea. It will be interesting to see whether such epigenetic analysis will supplant or accompany the current immunological tests for fecal blood. Hypermethylation of CpG islands in gene promoters is an epigenetic mechanism to repress transcription (Weber et al. 2007). It would therefore appear that syndecan-2 expression is downregulated in colon carcinoma, at least as far as serum and lumenal colon samples are concerned. However, this is most likely not as simple as it appears at first sight. As noted above, syndecan-2 may function to enhance the invasive potential of colon carcinoma cells, which suggests that perhaps the proteoglycan is expressed in the metastatic or premetastatic niche. There is clearly much more to learn regarding the regulation of syndecan-2 expression in this disease.

4.7 Conclusions

Colorectal cancer is the third most common malignancy and fourth leading cause of cancer-related death worldwide. As with many cancers, early detection can be an essential component of successful treatment. While syndecans have not been an intensively studied family of transmembrane receptors so far, it is fascinating to consider that syndecan-2 may not only be highly relevant to tumor progression, but epigenetic alterations in its methylation status may be a very useful tool for early

diagnosis. Syndecan-1 is now known to be a key receptor in the progression of myeloma and is a target for therapy (Yang et al. 2007; Jagannath et al. 2019; Schönfeld et al. 2017; Yu et al. 2020). Therefore, two of the four syndecans are connected strongly to cancers. Given that other ongoing work connects syndecans of invertebrates and vertebrates with stem cell regulation (Nakato and Li 2016; Gopal et al. 2016; Kumar Katakam et al. 2021), for example, through Notch signaling (Pisconti et al. 2010; Zhao et al. 2012), it is likely that increased relevance of syndecans to human health and disease will emerge. The linkage between syndecan function and their regulation of TRPC calcium channels (Gopal et al. 2015, 2016; Mitsou et al. 2017) is also very likely to impact cell behaviors. Therefore, even though there are little data to link syndecan core protein mutations with disease, the relevance of this family, with its long evolutionary history, both for normal and pathogenic tissues has justified their study.

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Chapter 5
Versican in Tumor Progression, Tumor–Host Interactions, and Cancer Immunotherapy

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Abstract Versican (VCAN) is a large matrix proteoglycan whose very name acts as a testament to its extensive versatility in tissue sculpting, inflammation, and cell fate specification. The broad spectrum of activity of the parent molecule is further diversified through the actions of VCAN's bioactive proteolytic products (VCAN–matrikines). This chapter, while focusing on the functions of VCAN in tumor progression and tumor immunity, contrasts VCAN's roles in adult tissue remodeling with VCAN's nonredundant functions in embryonic morphogenesis. We propose that the interplay between VCAN and VCAN–matrikines provides crucial survival, differentiation, and activation cues wherever tissue architecture is forged or altered, whether in the embryo or in the adult organism. Better understanding of VCAN's mechanisms in the last decade has led to the conceptualization and testing of novel

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biomarkers and therapies, particularly in the fast-paced field of cancer immunotherapy.

5.1 VCAN: Structure and Variant Forms

Versican (VCAN) is a large aggregating chondroitin sulfate (CS) proteoglycan and a member of the hyalectan family of matrix proteoglycans, which derive their name from their ability to bind hyaluronan (HA). Unlike many of its family members, such as aggrecan, neurocan, and brevican, VCAN is expressed in a variety of tissues throughout the body (Wu et al. 2005b). Its uniquely wide-spread expression predicts its vital, nonredundant roles in organ development and disease (Wight 2002). VCAN's importance is also exemplified by its highly conserved nature, with 89% of its amino acid sequence being identical between the human and mouse protein (Naso et al. 1995). The human gene for VCAN (*VCAN, CSPG2*) is encoded from a single locus on chromosome 5, comprises of 15 exons spanning over 90 kb of contiguous DNA, and is regulated by a typical TATA box, a promoter sequence between −209 and − 445 base pairs, and a negative regulatory sequence between −445 and − 632 base pairs (Iozzo et al. 1992; Wu et al. 2005b).

Characteristically for a hyalectan, VCAN displays globular motifs at its N-terminus (G1 domain) and C-terminus (G3 domain), bridged by a CS chain-binding region. The G1 domain is made up of an immunoglobulin (Ig)-like subunit followed by two HA-binding domains (link modules) (Fig. 5.1). VCAN's G3 domain is comprised of two epidermal growth factor (EGF)-like repeats, followed by carbohydrate recognition domain (lectin-like, CRD) and complement binding

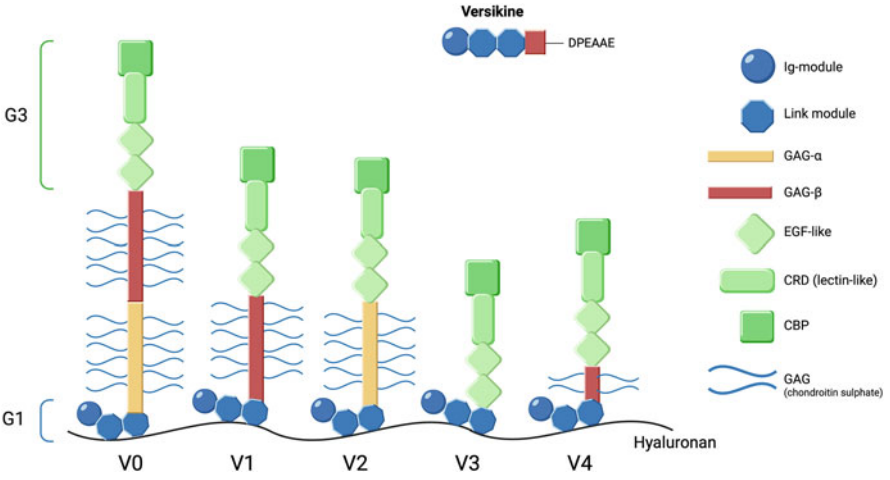

Fig. 5.1 Versican (VCAN) functional domains and splice isoforms. *CRD* carbohydrate recognition domain, *CBP* complement binding protein, *EGF* epidermal growth factor, *GAG* glycosaminoglycan, *Ig* immunoglobulin

protein (CBP)-like subunits. The CS-binding domain is further subdivided into glycosaminoglycan (GAG)-α and GAG-β regions (Wu et al. 2005b). Four common splice variants of VCAN have been identified: V0, V1, V2, and V3. These are distinct in the number and species of GAG regions present. The GAG-bearing domain of the VCAN core protein can be alternately spliced at exon 7 and exon 8, which code for the GAG-α and GAG-β regions, respectively. The V0 isoform is produced when neither exon 7 nor 8 are spliced out. When only exon 7 is spliced out, the V1 variant is generated, while V2 is formed when only exon 8 is spliced out. Translation of mRNA with both exons 7 and 8 spliced out results in VCAN V3. Since V3 is devoid of GAG regions, and thus also CS chains, it cannot technically be considered a proteoglycan. Despite this, it is frequently grouped with proteoglycans and characterized as such (Wight 2002, 2017). An additional isoform, V4, consisting of the G1 domain, the first 398 amino acids of the GAG-β-region and the G3 domain, has been detected in breast cancers (Kischel et al. 2010).

5.2 VCAN Binding Partners in the Tumor Microenvironment

VCAN engages binding partners both in the extracellular matrix and on the surface of cells in the tumor microenvironment. This section will cover key players that VCAN binds to, in both of these compartments, grouped by the domain of VCAN that they interact with.

The G1 region of VCAN is involved with binding hyaluronan and link protein (Matsumoto et al. 2003). Link protein has also been found to bind to other hyalectans like aggrecan in cartilage matrices. It plays an important role in connecting and building the scaffolding of extracellular matrix. Hyaluronan is a polysaccharide made up of 200 to 10,000 repeating units of the disaccharide β1,3 N-acetylglucosaminyl-β1,4 glucuronide (Toole 1990). While hyaluronan plays a role in the architecture of the extracellular matrix, it is also thought to be involved with cell signaling. A signaling role is suggested by its relatively high turnover rate for a matrix molecule—its half-life ranges from half a day to a few days—and its increased expression at sites of high cell activity such as angiogenesis, hematopoiesis, limb-bud formation, inflammation, and tissue remodeling (Laurent and Fraser 1992; Nandadasa et al. 2021a). Of the domains that make up the G1 region, the HA-binding regions are the most crucial, as they are necessary and sufficient for binding both HA and link protein. However, VCAN's binding affinity for both HA and link protein was shown to increase when the Ig-like region is present (Matsumoto et al. 2003).

Whereas the G1 region of VCAN is known for binding extracellular matrix molecules, the CS-binding domain is known for binding cell surface molecules. VCAN's GAG regions do not form extrinsic interactions through the protein backbone itself but through the branching chondroitin sulfate chains bound to the core

protein. For example, VCAN's ability to bind L- and P-selectins (cell adhesion molecules) is lost when VCAN undergoes chondroitinase digestion or is treated with blocking antibody (Kawashima et al. 2000). VCAN also binds chemokines via its GAG chains. Secondary lymphoid tissue chemokine (SLC) was shown to have a specific affinity for GAG-β and was significantly less efficient at inducing $\alpha_4\beta_7$ integrin activation the presence of VCAN (Hirose et al. 2001). CD44 has a few similarities to VCAN: both are chondroitin sulfate proteoglycans that bind to HA. CD44, in contrast to the secreted VCAN, is a transmembrane molecule, and much of its functionality is derived from its ability to endocytose and degrade HA (Hua et al. 1993). CD44 functions to mediate, among other things, angiogenesis, and cell migration out of the bloodstream into inflamed tissues (Lokeshwar et al. 1995; Savani et al. 2001). These combine to contribute to tumor progression and metastasis. A common theme of the aforementioned CS-binding cell surface molecules appears to be some involvement with leukocyte trafficking.

The G3 domain of VCAN is the most versatile region of the molecule. It has binding partners in both the extracellular matrix and on the cell surface. VCAN's EGF-like tandem repeats in its G3 region bind to the extracellular molecules fibulin-1 and -2, fibrillin, as well as the cell surface molecule P-selectin glycoprotein ligand-1 (PSGL-1). Fibrillin-1 is a predominant extracellular matrix molecule, and its epidermal growth factor-like domains 11 and 21 are likely where VCAN binds to microfibrils. Disruptions of these domains are associated with Marfan disease and can cause cardiovascular disease (Isogai et al. 2002). Outside of cardiovascular tissues, VCAN and fibrillin are also colocalized in blood vessel and dermal tissues (Wu et al. 2005b). These highly ordered and elastic structures also heavily feature fibulin, and may function as a bridge between VCAN and fibrillin (Wight 2002). Fibulin-1 and fibulin-2's calcium-binding EGF-like domains have been shown to be the ligand for VCAN's EGF-like tandem repeats (Aspberg et al. 1999).

P-selectin glycoprotein ligand-1 (PSGL-1) is a homodimeric cell surface molecule that is nearly ubiquitously expressed in the blood leukocyte compartment. The process of inflammation and tissue infiltration by immune cells begins with circulating cells sticking to the endothelium of blood vessels, followed by leukocyte rolling. It is this action of leukocyte rolling that PSGL-1 is involved with. When various version G3 constructs were exposed to PSGL-1, it was found that versions EGF-like tandem repeats and CRD motifs bound PSGL-1. The presence of the CBP motif suppressed their ability to bind PSGL-1 individually, but when both the tandem repeats and CRD were present, they resisted the CBP negative binding effect (Zheng et al. 2004a).

As previously mentioned, the CRD motif of VCAN's G3 binds PSGL-1. Its other primary binding partner is tenascin-R. This is a commonality in the lectican family, as brevican, neurocan, syndecan, and others bind to tenascin (Aspberg et al. 1997; Grumet et al. 1994; Salmivirta et al. 1991). Tenascin-R is expressed in the central nervous system, and it has been proposed to play a role in the macromolecular organization of the adult brain extracellular matrix based on its binding properties (Pesheva et al. 1991). The ultimate goal of the resulting matrix architecture,

including HA and lecticans such as VCAN, is to create a physical barrier around neural tissue, nonpermeable to other cells (Yamaguchi 2000).

The most C-terminal motif of VCAN's G3, the CBP domain, does not appear to have as many binding partners as the tandem repeats or CRD domains. One such partner, however, is fibronectin (Wu et al. 2005b). Fibronectin is a component of the extracellular matrix that is also an integrin ligand and has been shown to increase cell adhesion. VCAN reduces fibronectin's ability to adhere to pancreatic cancer cell lines, with this effect being diminished when VCAN is treated with chondroitinase, implying that VCAN's GAG chains play a part in this function (Wu et al. 2005b). Somewhat paradoxically, despite acting to limit fibronectin's ability to bind tumor cells in vitro, it has been suggested that VCAN's G3 domain may simultaneously upregulate expression of fibronectin in vivo to stimulate tumor propagation and angiogenesis (Zheng et al. 2004a). Finally, VCAN and fibronectin can complex along with VEGF to increase endothelial cell adhesion. Preventing the formation of the complex with an antibody against fibronectin abrogated G3's ability to induce this behavior (Wu et al. 2005b).

VCAN can bind to integrin β1, although it is not clear specifically which motif of the VCAN's G3 domain is involved (Wu et al. 2002). Integrin is a cell surface receptor and anchoring protein comprised of noncovalently linked heterodimer α and β subunits, each of which has several isoforms. The various αβ dimers have different binding specificities and cell signaling characteristics, including but not limited to, tumor initiation, proliferation, survival, and therapeutic resistance (Cooper and Giancotti 2019). Integrin β1 is also able to colocalize with EGFR to alter its downstream signaling pathways (Yamada and Even-Ram 2002). When the pheochromocytoma PC12 cell line was transfected to produce VCAN V1, it led to increased EGFR and integrin expression as well as PC12 neuronal differentiation and neurite outgrowth. This effect was significantly abrogated by inhibition of EGFR, integrin, or Src, a downstream cell signal. Thus, VCAN appears to alter cell differentiation via signaling through both integrin and EGFR pathways (Lee et al. 2015).

5.3 Post-Translational Modifications, Proteolysis, and VCAN–Matrikines

VCAN is cleaved by a disintegrin-like and metalloproteinase with thrombospondin-1 motifs (ADAMTS) and matrix metalloproteinase (MMP) enzymes to generate bioactive fragments, *VCAN–matrikines* (Sandy et al. 2001; Westling et al. 2004; Jonsson-Rylander et al. 2005). Matrix metalloproteinase MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 from the proteoglycan-cleaving superfamily degrade VCAN in vitro (Perides et al. 1995; Passi et al. 1999; Halpert et al. 1996). Plasmin degrades VCAN core protein in vitro (Kenagy et al. 2002). Six ADAMTS family proteinases (ADAMTS-1, −4, −5, −9, −15, and − 20) (Apte 2009) (Apte 2020; Mead and Apte

2018) are known to cleave VCAN, hence they are named "versicanases" (Nandadasa et al. 2014). Interestingly, ADAMTS-8 has also potential of cleaving VCAN since it shares significant protein homology with a subgroup of ADAMTSs that includes ADAMTS-1, −4, −5, and −15 and has been demonstrated to cleave "aggrecanase-susceptible" sites within the extracellular matrix (ECM) proteoglycan aggrecan (Collins-Racie et al. 2004). While VCAN core protein undergoes cleavage by different proteinases, regulated ADAMTS cleavage at specific sites is important for development and disease (see below).

Most of the characterized VCAN–matrikines contain the HA-binding G1 domain. The best-studied is generated by cleavage of a Glu^{441}-Ala^{442} bond (V1 enumeration) in the GAG-β domain of V0/V1, generating a neoepitope, DPEAAE (Sandy et al. 2001) and can be detected with an anti-DPEAAE antibody (Fig. 5.1). This proteolytic event generates a G1-$DPEAAE^{441}$ fragment of ∼80 kDa from the V1 isoform and a G1-$DPEAAE^{1428}$ fragment of ∼220 kDa (Sandy et al. 2001) from the V0 isoform. The G1-$DPEAAE^{441}$ fragment is named "versikine" and has roles in various cellular processes and in the regulation of immune phenotypes in the inflammatory milieu of tumors (Papadas et al. 2020; Hope et al. 2016; Nandadasa et al. 2014; Kern et al. 2006; Kern et al. 2007). The mode of action of versikine has not been fully determined yet. Since it derives from the V1 variant, versikine consists of the G1 fragment and a short stretch (∼94 amino acid residue) of GAG-β. Despite the known function of the G1 domain in binding HA and expanding HA-rich cellular glycocalyx, whether versikine solely acts through binding to HA has not yet been definitively determined (Islam and Watanabe 2020). MMP-8 and -12 cleave N-terminally at position 3306 to generate the neoepitope KTFGKMKPRY found at atherosclerotic plaques in individuals with varying degrees of atherosclerosis (Barascuk et al. 2013). High performance liquid chromatography and sequence analysis demonstrated that ADAMTS-1 cleaves V0/V2 at Glu^{950}-Gly^{951} and V0/V1 at Tyr^{1410}-Ile^{1411} and Tyr^{423}-Ile^{424} in vitro, but the in vivo physiological consequences of these events are unclear (Jonsson-Rylander et al. 2005). Glial hyaluronate-binding protein (GHAP), found mainly in the brain ECM, is a 64 kDa HA-binding fragment, which is generated through cleavage of Glu^{405}-Gln^{406} bond on V0/V2 by ADAMTS-4 (Westling et al. 2004). Immunological analysis of purified human GHAP showed that it contained the C-terminal sequence, $NIVSFE^{405}$, and arose as a product of cleavage and digestion of human cerebellum VCAN with ADAMTS-4. Besides the Glu^{405} fragment, which was the major immunoreactive species, at least six other C-terminally truncated V2 fragments of varying GAG-α domain length were identified in brain tissue (Westling et al. 2004).

Multiple more cleavage sites have been identified or predicted but whether they generate activities that impact on their tissue and organ microenvironment is unknown (Hope et al. 2016). There is undeniable evidence that certain VCAN–matrikines can act as key drivers of cell-fate determination, immunity, and inflammation in a variety of physiological and disease processes (Mushtaq et al. 2018). However, their overall effect seems to be context-dependent.

5.4 VCAN and VCAN–Matrikines in Development

VCAN and its ADAMTS-generated fragments play a crucial role in cardiovascular, neural crest cell migration, and skeletal development (Papadas and Asimakopoulos 2020). VCAN's proteolytic processing is essential for morphogenesis at sites such as interdigital webs, secondary palate shelves prior to the midline fusion, myocardial contraction, and the fashioning of mature heart leaflets (Nandadasa et al. 2014). VCAN is much more prevalent in embryonic than adult ECM, except for brain tissue. VCAN positively regulates the formation of a mesenchymal matrix, ideal for cell invasion, migration, and morphogenesis in the embryo (Kamiya et al. 2006). As also discussed later in this chapter with regard to adult tissues, the spectrum of activities of parental VCAN and its proteolytic products in embryonic tissues often does not overlap and in some cases, could be even characterized as antagonistic (Nandadasa et al. 2014).

VCAN is essential for cardiac development, contributing to the formation of endocardial cushion mesenchyme by epithelial–mesenchymal transformation (EMT) (Kern et al. 2006). Altered ADAMTS expression plays a crucial role in the stoichiometric balance of intact versus cleaved VCAN in the stroma and overall ECM structure and organization of the developing heart. VCAN proteolytic fragments generated through the actions of ADAMTS proteases can be detected in the cardiac cushions. Endocardial cushions are then promptly remodeled to reach structural maturity and VCAN fragments are distributed around cushion mesenchymal cells. Kern and colleagues reported that *Adamts9*-haploinsufficient and *Adamts5*-null mice had intact VCAN accumulation in the mitral valves, which led to congenital valve anomalies due to defects and alterations in extracellular matrix remodeling (Kern et al. 2007). Intact VCAN accumulated also in the aortic and pulmonary artery walls and was associated with several pathologies including increased adventitial thickness, interruption of the aortic wall and reduction of aortic interlamellar elasticity (Kern et al. 2010). Myocardial trabeculation is another crucial context of VCAN–matrikine activities (Stankunas et al. 2008). During trabeculation, both myocardial and endothelial cells must undergo extensive cellular movements to form the long thin projections protruding into the ventricular cavity (Stankunas et al. 2008). Thus, establishment of a favorable extracellular milieu that promotes changes in migration, cell shape, and adhesion may be critical for trabeculation. In particular, the upregulation of ADAMTS-1 caused VCAN reduction and premature breakdown of the cardiac jelly as well as termination of trabeculation. Contrarily, trabeculation was rescued in *Adamts1*-null mice, as the ventricles of the latter exhibited increased trabecular muscle compared to wild type (Stankunas et al. 2008). Loss of fibulin-1, an ADAMTS-1 cofactor, also led to diminished VCAN cleavage and increased trabecular growth (Cooley et al. 2012). The equilibrium between intact and cleaved VCAN is a central regulator of trabeculation, and ADAMTS-1 activity is suppressed by endocardial Bgr1, a chromatin-based transcriptional regulator, until trabeculation is completed (Stankunas et al. 2008). Finally, immunohistochemical studies demonstrated colocalization of VCAN accumulation and degradation in developing

mouse lungs as detected by antibodies against intact VCAN and DPEAAE, respectively, suggesting functional roles of VCAN deposition and degradation in lung morphogenesis (Snyder et al. 2015).

VCAN proteolysis by ADAMTS-9 in vascular endothelium and by ADAMTS-20 in palate mesenchyme results in palatal shelf sculpting and extension. Co-ordination of ADAMTS-9 and ADAMTS-20 contributes to secondary palate closure (Enomoto et al. 2010). Mesenchyme of *Adamts9*$^{+/-}$ and *Adamts20*$^{bt/bt}$ mutant murine palatal shelves had reduced cell proliferation, lower cell density, and decreased processing of VCAN. *Vcan* haploinsufficiency in the *Adamts20*$^{bt/bt}$ background produced cleft palate, providing proof that VCAN is a ADAMTS partner during palate closure, possibly operating through versikine (Enomoto et al. 2010). VCAN and hyaluronan are also associated with emerging Flk1^{+} hematoendothelial progenitors at gastrulation. Apte and colleagues recently demonstrated that the mouse VCAN mutant *Vcan*^{hdf} lacked yolk sac vasculature, with attenuated yolk sac hematopoiesis. In addition, hyaluronan was severely depleted in *Vcan*^{hdf} embryos. Conversely, hyaluronan-deficient mouse embryos also had vasculogenic suppression but with increased VCAN proteolysis. $VEGF_{165}$ and Indian hedgehog, crucial vasculogenic factors, utilized the VCAN–hyaluronan matrix, specifically VCAN chondroitin sulfate chains, for binding. VCAN–hyaluronan ECM is thus essential for vasculogenesis and primitive hematopoiesis (Nandadasa et al. 2021b).

VCAN cleavage may involve sequential events (Montgomery et al. 1993; Arner 2002), first generating G1 and GAG-β-G3 fragments, followed by the loss of GAG-β and CS chains, freeing globular domains that may persist indefinitely (Kenagy et al. 2005, 2006). The initial cleavage site may be the site generating versikine from the V1 isoform (corresponding to Glu^{1428}-Ala^{1429} in V0, and Glu^{441}-Ala^{442} in V1 and V4 variants in humans, respectively). The Watanabe group evaluated the relevance of the initial cleavage site in VCAN turnover by generating knock-in mice, V1R, whose VCAN is mutated at the initial cleavage site in V0 and V1 (Islam et al. 2020). V1R homozygote (R/R) mice showed fewer littermates. When studied, some embryos were small, and the others exhibited organ hemorrhage and syndactyly (Islam et al. 2020). The presentation of hindlimbs and fused digits was consistent with the earlier Apte data implicating VCAN proteolysis in interdigital web regression (McCulloch et al. 2009). More recently, the Apte group reported a new mouse transgene, *Vcan*^{AA}, with validated mutations in the GAG-β domain that specifically abolishes the generation of versikine (Nandadasa et al. 2021a). As a result, *Vcan*$^{AA/AA}$ mice presented with partially penetrant hindlimb soft tissue syndactyly. However, *Adamts20* inactivation in *Vcan*$^{AA/AA}$ mice led to fully penetrant, more severe phenotype affecting all limbs, suggesting that ADAMTS-20 cleavage of VCAN at other sites or of other substrates is an additional requirement for web regression. Interestingly, mice with deletion of *Vcan* exon 8, encoding the GAG-β domain, consistently developed soft tissue syndactyly, while mice lacking exon 7, encoding the GAG-α domain in Vcan transcripts, consistently had fully separated digits. These findings suggest that VCAN is cleaved within each GAG-bearing domain during web regression but generation of versikine through proteolysis in the GAG-β domain retains essential role in interdigital web regression (Nandadasa et al. 2021a).

5.5 VCAN in Tumor Progression and Metastasis

VCAN supports tumor growth and facilitates metastatic dissemination. Increased VCAN expression has been described in a wide variety of malignant tumors, and it correlates with both cancer relapse and poor patient outcomes (Papadas and Asimakopoulos 2020). There are diverse sources of VCAN production in the tumor microenvironment: the tumor cells, the stromal cells, the tumor-associated myeloid cells, and possibly lymphoid cells in some contexts. In lung cancer, VCAN's principal source of secretion appears to be the tumor cell (Kim et al. 2009). Tumor cells also show an elevated expression of VCAN in ovarian cancer (Li et al. 2013), leiomyosarcoma (Keire et al. 2014), hepatocellular carcinoma (Xia et al. 2014), colon carcinoma (Bögels et al. 2012), glioma (Hu et al. 2015), and bladder cancer (Said and Theodorescu 2012). In other contexts, stromal cells constitute the main source of VCAN production, such as in prostate cancer (Ricciardelli et al. 1998; Sakko et al. 2003; Sakko et al. 2001), ovarian cancer (Yeung et al. 2013), pharyngeal cancer (Pukkila et al. 2004), breast cancer (de Lima et al. 2012; Kischel et al. 2010; Ricciardelli et al. 2002; Takahashi et al. 2012), and colon cancer (Iozzo 1995). In some tumors, the source of VCAN could be both the stromal and tumor cells. The combination of tumor and stromal expression of VCAN correlates with shortened disease-free survival and overall survival (Kodama et al. 2007). Moreover, in spontaneous breast cancer murine models, $CD11b^{+}Ly6C^{high}$ monocytic cells are the main producers of VCAN (Gao et al. 2012), whereas in Sezary syndrome, a leukemic variant of cutaneous T cell lymphoma, VCAN was identified as one of the most upregulated genes in lymphocytes isolated from patients (Fujii et al. 2015).

VCAN plays a central role in almost each and every hallmark of cancer (Hanahan and Weinberg 2011). Each of the four functional domains/motifs has been implicated in diverse cancer-promoting processes. The precise VCAN domain/motif that influences tumor cell behavior may be dependent on the specific context and microenvironment (Islam and Watanabe 2020; Wight et al. 2020). VCAN is an important player in tumor cell proliferation and self-renewal: the G1 domain of VCAN has been demonstrated to promote proliferation by interrupting cell adhesion, whereas the G3 domain promotes proliferation through two EGF-like motifs, which are implicated in triggering cell growth. VCAN V0/V1 interacts with TGF-β2 and stimulates proliferation and migration of high-grade gliomas. Silencing of VCAN-isoform V1 by siRNA reduced tumor cell proliferation in human glioma cells (Onken et al. 2014). The G3 domain of VCAN interacted with beta-1 (β1) integrin, activated focal adhesion kinase, enhanced integrin expression, and promoted cell adhesion in glioma cells rendering them resistant to free radical-induced apoptosis (Wu et al. 2002, 2005a). Finally, coexpression of G1 and G3 domains of VCAN protects cells from apoptosis induced by death receptor ligands or cytotoxic drugs (Cattaruzza et al. 2004).

VCAN has been implicated in the formation of new blood vessels in nascent tumors. Tumor neo-angiogenesis provides developing tumors with adequate oxygen

supply and nutrients. In Lewis Lung Carcinoma (LLC) tumors, stromal cell-derived VCAN and versikine are associated with increased angiogenesis (Asano et al. 2017). A significant regression of tumor volume as well as capillary formation in the *Vcan*$^{hdf/+}$ mice at 10 days and 13 days post-tumor inoculation compared to wild-type mice was observed (Asano et al. 2017). Accumulation of VCAN in tumors is also positively correlated with the number of microvessels within tumor stroma (Labropoulou et al. 2006; Ghosh et al. 2010). For instance, human stromal stem cells that produce elevated levels of VCAN were found to form an extensive vascular network enriched in hyaluronan and VCAN when cultured with vascular endothelial cells (Kreutziger et al. 2011). In the context of a highly vascularized tumor, glioblastoma, VCAN also appears to promote angiogenesis. The VCAN G3 domain augmented angiogenesis both in vitro and in vivo. G3-expressing cells and tumors formed by these cells expressed very high levels of fibronectin and VEGF. Moreover, the G3 domain after interacting with fibronectin carved a complex together with VEGF. This complex promoted vascular formation in endothelial cells, which lined the vessel walls in human astrocytoma tumors (Zheng et al. 2004b). Consistent with the finding that G3 domain binds fibronectin, the V2 VCAN isoform enhanced substantial blood vessel formation by upregulating and binding to fibronectin (Yang and Yee 2013). Silencing fibronectin expression by siRNA abrogated V2 VCAN's effect in promoting tube-like vascularization (Yang and Yee 2013).

VCAN promotes local tumor cell motility and invasion (Ricciardelli et al. 2009). The G1 and G3 domains seem to exert different effects in cancer cell motility. VCAN enhances cancer cell migration and reduces cell adhesion through its G1 domain. VCAN stimulates locomotion and reduces cell adhesion of astrocytoma cells through binding of its G1 domain to HA and link protein (HAPLN1) (Ang et al. 1999; Yang et al. 1999). On the other hand, VCAN G3 domain appears to be more important in local and systemic tumor invasiveness of breast cancer (Yee et al. 2007). Moreover, VCAN proteolysis plays an important role in cancer cell motility and invasion via signaling pathways in the tumor microenvironment. Arslan and his colleagues demonstrated in immunochemical analyses of glioma tumors that exogenous TGF-β2 not only induced V1 expression but also increased MMP-2 activity and the migration rate of glioma cells (Arslan et al. 2007). However, treatment of glioma tumor spheroids with a blocking anti-DPEAAE antibody reversed the enhancement of glioma migration induced by TGF-β2 in a dose-dependent manner. Thus, VCAN proteolysis facilitates TGF-β2's effect on tumor cells (Arslan et al. 2007). In addition, hyaluronectin, a glycoprotein with an amino acid sequence corresponding to nucleotides of 1 to 1372 of the human VCAN N-terminus, was shown to exert opposing effects on lung cancer cell metastatic spread depending on its primary tumor concentration: elevated levels reduced migration in vitro and metastasis in vivo, while low levels boosted migration and metastasis (Paris et al. 2006). The formation of an HA–VCAN pericellular matrix augmented prostate cancer motility in Boyden chamber motility assays using fibronectin as a chemoattractant. Thus, prostate cancer cells in vitro have the ability to recruit ECM components, such as VCAN, secreted by prostatic stromal cells to promote their motility (Ricciardelli et al. 2007). This observation is consistent with a scenario

whereby the formation of a pericellular sheath in vivo by prostate cancer cells utilizing VCAN laid down by prostate stromal cells may contribute to the development of metastatic disease. In glioma, VCAN V0 and V1 expression in tumor vessels was increased and was described to promote local invasion (Paulus et al. 1996). In chondrosarcoma, V1 isoform transfection significantly enhanced tumor cell motility and migration (Wasa et al. 2012). Elevated VCAN expression in tumor-associated stroma also promoted local invasion in cervical cancer and decreased the numbers of intraepithelial CD8+ T cells (Gorter et al. 2010), whereas increased expression of CD44 and VCAN correlated with reduced expression of E-cadherin in endometrial cancer (Hanekamp et al. 2003). Finally, HA oligomers were found to impede the formation of pericellular matrix by ovarian cells and the increased motility and invasion triggered by recombinant VCAN. Therefore, HA oligomers could be a potential adjuvant therapeutic modality, administered intraperitoneally together with chemotherapy drugs, after surgical ovarian cancer debulking (Ween et al. 2011).

VCAN accumulation is associated with tumor metastasis to distant organs. Michael Karin and colleagues have showed that by activating TLR2/TLR6 complexes and inducing TNF-α secretion by myeloid cells, VCAN strongly enhanced metastatic growth in the murine lung cancer model, Lewis Lung Carcinoma (LLC). Metastatic enhancement was abrogated in *Tlr2*$^{-/-}$ mice (Kim et al. 2009). TNF-α is one of the main pro-metastatic factors secreted by host myeloid cells. TNF-α can repress the apoptosis of cancer cells and promote their proliferation through NF-κB activation (Luo et al. 2004). Moreover, by increasing vascular permeability, TNF-α can augment recruitment of leukocytes as well as intravasation and extravasation of cancer cells. In mouse mammary tumor virus-polyoma middle T-antigen (MMTV-PyMT)-induced cancers, VCAN produced from CD11b^{+} Ly6C^{high} monocytes was essential in advancing metastasis to the lung in a TGF-β-dependent manner (Gao et al. 2012). Consistently, VCAN knockdown in the bone marrow significantly impaired lung metastases in vivo, without impacting their recruitment to the lungs or altering the immune microenvironment (Gao et al. 2012). In the 4 T1 murine mammary carcinoma model, VCAN expression in the lung correlated with tumor-associated macrophage (TAM) abundance and higher numbers of pulmonary metastatic nodules (Dos Reis et al. 2019). Finally, Mitsui and colleagues highlighted the oncogenic role of VCAN in clear cell carcinoma by demonstrating that VCAN expression was upregulated in patients with clear cell renal carcinoma (ccRCC), and this upregulation was associated with adverse patient outcomes and high rate of metastasis. In a study of 84 matched sporadic ccRCC and normal renal tissues, patients with high VCAN expression had a significantly worse 5-year OS (overall survival) (p-value = 0.007) and a higher rate of systemic metastasis than those with low VCAN expression (p-value = 0.0139). VCAN significantly promoted ccRCC cell migration and invasion through MMP7 and CXCR4 induction (Mitsui et al. 2017).

5.6 VCAN in Tumor Inflammation and Antitumor Immunity

VCAN is produced by, and conversely regulates, crucial components of the myeloid regulatory cell network in the tumor microenvironment (Wang et al. 2009; Tzanakakis et al. 2019). In particular, several key studies in the last decade demonstrated VCAN's role in controlling the function of intratumoral dendritic cells and macrophages. Dendritic cells (DCs) are professional antigen-presenting cells that fine-tune immune responses and help determine the balance between immunity versus tolerance. With regard to tumor contexts in particular, the important cross-presenting Batf3-DC (also known as conventional dendritic cell type 1, cDC1) subset has been shown to be essential for intratumoral effector T cell response and immunotherapy efficacy (Spranger et al. 2017). Batf3-DC are indispensable for "innate sensing" of tumors and priming of the adaptive immune response (Merad et al. 2013). However, Batf3-DC can be mediators of tolerogenic adaptive immune responses in different contexts. It is interesting that VCAN, through its multifaceted activities, can alternatively induce tolerogenic vs. immunogenic polarization of tumor DC.

Tumor-derived VCAN leads to DC dysfunction (tolerogenic polarization) through TLR2 activation. TLR2 ligation has dual consequences: first, secretion of autocrine IL-10 and IL-6, and second, enhanced expression of their cytokine receptors, which decreases the threshold required to activate STAT3. This positive feedback loop renders DCs tolerogenic which results in blunted antitumor responses through priming of regulatory or ineffective T cells. Thus, VCAN–TLR2 signaling impedes immunogenic DC maturation and limits Th1 and cytotoxic lymphocyte (CTL) responses (Tang et al. 2015, 2017).

By stark contrast, immunogenic DC polarization can be induced through VCAN's proteolytic fragments, VCAN–matrikines (the best studied among them being versikine) (Papadas et al. 2021). Matrikines have been defined as extracellular matrix-derived fragments that regulate cell activity, often in a manner distinct from that of their parent macromolecule (Maquart et al. 2004). VCAN proteolysis at Glu^{441}-Ala^{442} is associated with robust CD8+ infiltration in multiple myeloma bone marrow (Dhakal et al. 2019; Hope et al. 2016) as well as solid tumors (Hope et al. 2017). This specific proteolytic event can produce CD8+ infiltration through either local VCAN depletion or the generation of versikine (or both). Indeed, a role for VCAN depletion in independently regulating immune cell influx into tumors would seem to be supported by earlier data showing that reduced stromal VCAN correlated with immune effector infiltration in cervical cancer biopsies (Gorter et al. 2010). However, there is strong emerging evidence to suggest that versikine, far from being an inert remnant of proteolysis, may constitute the more crucial entity responsible for CD8+ T cell mobilization. Consistent with this scenario, versikine triggers type I interferon-dependent transcription in myeloid cells (Hope et al. 2016). In addition, versikine promotes IRF8-dependent Batf3-DC (Grajales-Reyes et al. 2015; Murphy 2013) generation from Flt3L-mobilized BM in vitro (Hope et al.

2017) and Batf3-DC density in vivo (Papadas et al. 2019; Papadas et al. 2021). Whereas the precise mechanisms of versikine's actions in vivo remain to be elucidated, the available data suggest that versikine may regulate intratumoral CD8+ influx through regulation of abundance, and activation of Batf3-DC (Papadas et al. 2021). It is interesting that versikine appears to act a tug-of-war with its parent macromolecule, nonproteolyzed VCAN, with regard to CD8+ infiltration. Whereas most matrikines possess activities that merely differ from those of their parent macromolecules, versikine appears to actively antagonize its parent.

The role of VCAN and VCAN–matrikines in macrophage regulation is also well established, both in the context of tumor and non-neoplastic inflammatory conditions. VCAN acts as a danger-associated molecular pattern (DAMP) molecule that interacts with Toll-like receptors (TLRs), such as TLR2 on alveolar macrophages, to promote production of inflammatory cytokines, including tumor necrosis factor-α (TNFα), IL-6, and other pro-inflammatory cytokines (Gill et al. 2010; Wight et al. 2014; Wang et al. 2009; Kim et al. 2009). LPS and poly I:C, two TLR agonists, stimulate VCAN expression in both murine bone marrow-derived macrophages in vitro and in murine alveolar macrophages (Chang et al. 2014, 2017) as well as in stromal cells in vivo (Kang et al. 2017). In mesothelioma, VCAN enhances tumor progression through pro-tumoral macrophage polarization. Mice harboring VCAN-deficient tumors presented fewer tumor/pleural macrophages and neutrophils, and fewer pleural T-regulatory cells, compared to control animals (Pappas et al. 2019). In multiple myeloma (MM), VCAN is robustly expressed and processed in the bone marrow (Hope et al. 2014). We previously proposed a model in which VCAN activates myeloma-associated monocytes/macrophages through TLR2/6 signaling, thus triggering trophic IL-1β and IL-6 upregulation (Hope et al. 2014). A role for the VCAN pathway in human MM has been highlighted by two recent reports: Bailur et al. produced a high-resolution analysis of the human immune microenvironment in MM showing that myeloid-derived VCAN transcription was correlated with MM progression, loss of protective T cell stem-like (Tcf1+) memory and expansion of dysfunctional/exhausted T effectors (Bailur et al. 2019). Moreover, the Paiva group showed that immunosuppressive macrophages (expressing VCAN, ENTPD1, and STAB1) were associated with persistence of minimal residual disease (MRD) postautologous stem cell transplant for MM, and higher probability of relapse (Arana et al. 2016). Additional levels of complexity of VCAN's actions have been suggested through elegant studies by the Wight group, using VCAN conditional-null mice: VCAN produced by myeloid cells can have a distinct spectrum of activity compared to stromal-derived VCAN in a model of lung inflammation (Wight et al. 2020). Similar studies in tumor models are not yet available but should yield exciting insights about the diversity of VCAN's actions on the immune microenvironment.

It is important to note that versikine is likely only one of multiple bioactive VCAN–matrikines, as noted earlier in this chapter within the context of embryonic development. VCAN–matrikines other than versikine have been reported, but are much less-well studied. A proteolytic fragment of VCAN detected in neural tissue, glial hyaluronan-binding protein (GHAP) may possess immunomodulatory activities (Bignami et al. 1993). GHAP is a matrikine generated through ADAMTS

proteolysis of VCAN-V0 and -V2 at a consensus cleavage site (Glu^{405}-Gln^{406}) distinct from the site cleaved to release versikine (Westling et al. 2004). In preliminary unpublished work from the Asimakopoulos laboratory, GHAP could stimulate type I interferon-dependent transcripts in human macrophages. Further investigation is needed to unravel the full spectrum of VCAN activities through the compendium of VCAN–matrikines.

5.7 VCAN Biomarkers in Tumor Immunology and Immunotherapy

While VCAN appears to have a broad range of immunoregulatory effects in the tumor microenvironment, several publications have focused more specifically on the association between VCAN and tumor-infiltrating lymphocytes (TILs) in patient tumors. TIL abundance is an important characteristic within patient tumors, demonstrating the ability in different disease settings to predict disease progression, immune therapy response, and chemotherapy response (Shaban et al. 2019; Bai et al. 2020; Kong et al. 2018). However, TIL infiltration is not a direct measure of these cells' functionality, only their ability to migrate into tumors. Accordingly, patient tumors with high TIL infiltration do not respond to immune therapy equally and can have substantial heterogeneity in the functionality of effector populations (Paijens et al. 2021). This indicates the need for a biomarker that gives a more complete assessment of both immune infiltration and function. With regard to immune function, this chapter has already outlined several of VCAN's immunosuppressive functions; however, VCAN has also been shown to predict TIL infiltration in patient tumors. Together this indicates VCAN can be both a predictor of TIL functionality and infiltration, and possibly a better predictor of immune therapy response in patients.

The regulation of the abundance of TILs in tumors is multifaceted with the tumor microenvironment playing a very important role in this process. Recently, VCAN accumulation has been shown to inversely correlate with TIL abundance in a large cohort of colorectal cancer patient samples (Hope et al. 2017). In this study, it was shown that patient tumors with high levels of VCAN accumulation have significantly fewer TILs. In addition to the overall abundance of VCAN, the proteolysis of VCAN into versikine was shown to be a strong predictor of TIL abundance. Patient tumors with low levels of intact VCAN and high levels of versikine showed the highest average number of TILs. Similar analyses were also extended into breast, pancreatic, esophageal, and neuroendocrine tumors ($N = 1780$ in total) showing the same inverse trend between VCAN accumulation and the abundance of TILs within patient tumors (Emmerich et al. 2020). Two additional studies also identified the same inverse trend between VCAN accumulation and CD8+ T cell infiltration in both cervical cancer patient samples and post-ACST multiple myeloma core biopsies (Gorter et al. 2010; Dhakal et al. 2019). Altogether, these findings across several

cancer settings, including both solid and hematological, show that the accumulation of VCAN may be altering the tumor microenvironment to one that may be hindering either CD8+ T cell infiltration or function. These studies also demonstrate that VCAN proteolysis may have the opposite effect, correlating with higher levels of antitumor immune activation. At this point, it is unclear if the lack of TILs found in tumors with high levels of VCAN is the result of a direct or indirect mechanism. Future research is needed to evaluate this mechanism and whether or not it can be disrupted to improve treatment outcomes.

The lack of immune infiltration seen in many cancers is widely believed to be large contributor to immune checkpoint blockade therapy (ICB) resistance, and with VCAN demonstrating many immunosuppressive functions, it is possible VCAN accumulation or expression within tumors may be a marker for ICB resistance in patients. A recent study correlated the fraction of VCAN positive tumor-associated macrophages with patient's response to immune checkpoint blockade in melanoma, finding higher levels of VCAN+ macrophages in nonresponding patients (Cheng et al. 2021). An additional piece of evidence supporting VCAN as a biomarker for immune therapy response was found in a small phase II study comparing progression-free survival at 12 months between patients with high and low levels of VCAN accumulation or VCAN proteolysis (Deming et al. 2020). Prior to surgery, patients were treated with stereotactic body radiation therapy (SBRT) and anti-PD-1 therapy, followed postoperatively with several additional rounds of anti-PD-1 treatment. Patients with low-to-moderate levels of VCAN had a significantly lower rate of disease recurrence, with 67% of patients ($N = 12$) remaining disease-free at 12 months postoperatively. Conversely, both of the patients in this study with high levels of VCAN had disease recurrence by the same time-point. Additionally, an even stronger difference in recurrence-free survival at 1 year was seen between patients with high and low levels of VCAN proteolysis. Patients with high levels of VCAN proteolysis had significantly lower rates of disease recurrence at 12 months, with 80% of patients remaining disease-free, whereas only 44% of VCAN-proteolysis-weak (VPW) patients made it to 1 year without recurrence. Together these data indicate that VCAN's immunoregulatory properties may make it an excellent marker for ICB response or disease recurrence following SBRT and anti-PD-1 treatment. It is important to note, however, that the cohort for the latter study was small and did not directly correlate VCAN accumulation or proteolysis with response to ICB therapy. Future studies are currently underway to address these pitfalls in order to evaluate more directly if VCAN can predict patient response to ICB therapy.

5.8 Prospects for VCAN Therapeutics in the Immuno-Oncology Era

A decade following the initial reports of checkpoint inhibitor efficacy in common cancer types, most patients are still refractory (Sharma and Allison 2020; Waldman et al. 2020; Havel et al. 2019). Early attempts at elucidating the molecular and physiologic underpinnings of response pointed to the importance of an "inflamed' immune microenvironment, i.e., evidence of pre-existing adaptive antitumor immunity that could be potentiated through immunotherapy (Ochoa de Olza et al. 2020).

The role of tumor matrix in regulating key players in adaptive immunity has not received adequate scrutiny. By contrast, the notion that matrix components and their bioactive fragments can act as endogenous "alarmins" has been firmly established (Schaefer 2014). Small leucine-rich proteoglycans were among the prototypes of matrix components acting as danger-associated molecular patterns (DAMPs) to activate Toll-like receptors in settings of inflammatory tissue damage (Schaefer and Iozzo 2012). Of the large matrix proteoglycans, VCAN is perhaps best studied as a regulator and amplifier of inflammation in the adult.

The interplay between VCAN and VCAN–matrikines provides an exciting model to understand the impact of matrix remodeling in regulating the tumor "immune thermostat." VCAN and its proteolytic "daughters" actively antagonize each other in the framework of the tumor immune milieu. Whereas stromal nonproteolyzed VCAN promotes T cell exclusion, the bioactive fragment versikine promotes Batf3-DC recruitment, survival, and activation. Therefore, versikine helps sculpt an immune microenvironment where Batf3-DC are encouraged to home in, and whose homeostasis is locally supported. Versikine may be useful in updialing the immunogenicity of a given tumor, toward a more inflamed "hot" setpoint, thus lowering threshold for immunotherapy efficacy. Thus, versikine could be a useful agonist in in situ vaccination approaches either alone or in combination with locally administered immunotherapy.

The yin vs. yang of VCAN vs. versikine appears critical in the regulation of adaptive immune responses through the crucial Batf3-DC subset (Schmitt 2016). Boosting and/or activating Batf3-DC at the tumor site is a crucial requisite for efficacy of several modern immunotherapy modalities. Versikine could synergize with diverse contemporary immunotherapy approaches to boost endogenous or engineered responses, in both hematopoietic and solid cancers.

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Chapter 6
Circulating Proteoglycans/Glycosaminoglycans as Cancer Biomarkers

Antonio Junior Lepedda, Gabriele Nieddu, Nikos Karamanos, and Marilena Formato

Abstract Cancer propagation and progression are associated with remarkable remodeling of the extracellular microenvironment with the formation of a permissive matrix for tumor growth, enriched in inflammatory mediators and matrix-degrading enzymes. Proteoglycans, which represent major structural and functional extracellular matrix components, play pivotal roles by affecting tissue organization, cell–matrix interactions, cell signaling, and, ultimately, cell behavior in physiological conditions as well as during cancer development and progression. In this respect, the proteolytic activity in tumor stroma liberates bioactive fragments, which can be detected in serum and may be useful as diagnostic and prognostic markers. Furthermore, since cell surface proteoglycans are specifically expressed by cancer cells, as in the case of glypican-3 in hepatocellular carcinoma, they can represent potential promising targets for immunotherapy.

This chapter reviews and critically addresses the major clinical literature and provides an overview of the significance of circulating proteoglycans (PGs) and glycosaminoglycans (GAGs), especially their soluble forms originating from the cell surface heparan sulfate proteoglycans syndecans and glypicans, in both prognosis and diagnosis of various malignancies responsible for high mortality and disability, including hepatocellular carcinoma, multiple myeloma, breast cancer, and renal cell carcinoma.

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6.1 Introduction

Cancer is an ever-growing healthcare problem, being the second leading cause of death globally, with an estimated 9.6 million deaths in 2018 (https://www.who.int/health-topics/cancer). It is a group of related diseases caused by genetic mutations leading to an abnormal cell proliferation, which can potentially invade other parts of the body.

Tumor cells, together with inflammatory cells and activated resident fibroblasts or cancer-associated fibroblasts (CAFs), create a microenvironment enriched in growth factors, cytokines, and extracellular matrix (ECM)-degrading enzymes. ECM, which physiologically provides cells with the chemical and mechanical signals essential for maintaining tissue homeostasis, is a key player undergoing deep remodeling that, in turn, supports tumor growth and spreading (Yuzhalin et al. 2018). In this respect, proteoglycans (PGs), as major functional ECM components, are crucial for tumor cells–matrix interactions and modulation of the activity of soluble factors (Theocharis and Karamanos 2019).

PGs are a family of ubiquitously distributed macromolecules, composed of specific core protein moieties bearing one or more O- or N-linked sulfated glycosaminoglycan (GAG) chains, primarily responsible for their biological properties (Raman et al. 2005), which can also be found in body fluids, i.e., plasma and urine.

GAGs are a group of complex anionic and unbranched heteropolysaccharides composed of repeating disaccharide units containing an *N*-acetylated hexosamine, either an *N*-AcetylGalactosamine (GalNAc) or an *N*-AcetylGlucosamine (GlcNAc), and an hexuronic acid (D-Glucuronic acid (GlcA) or its carbon-5 epimer L-Iduronic acid (IdoA)) or, exclusively in keratan sulfate, Galactose. GAGs are key structural and functional components of the ECM of all connective tissues, being involved in numerous events including embryonic development, ECM assembly, and regulation of cell signaling in various pathophysiological conditions. Their structural heterogeneity, in terms of repeating disaccharide unit, chain length, degree and pattern of sulfation, as well as degree of epimerization, is such huge that, virtually, there are not two identical glycosaminoglycans in the body, and is responsible for most of the numerous biological functions of PGs (Karamanos et al. 2018). Indeed, it has been recently shown that GAGs may interact with more than 800 different proteins (Vallet et al. 2021), including both cytokines and chemokines, as well as enzymes and enzyme inhibitors, ECM proteins, and membrane receptors (Kjellen and Lindahl 2018). Because of their pleiotropic functions and physicochemical properties, GAGs have also been employed as therapeutic agents as well as in many tissue engineering applications (Lepedda et al. 2021a).

According to the type of repeating disaccharide unit, six main classes of GAGs have been described so far: hyaluronan or hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), and heparin (Hep) (Fig. 6.1).

PGs that can be found in the ECM include versican (belonging to the hyalectans subfamily), decorin, and biglycan (belonging to the small leucine-rich PGs (SLRPs)

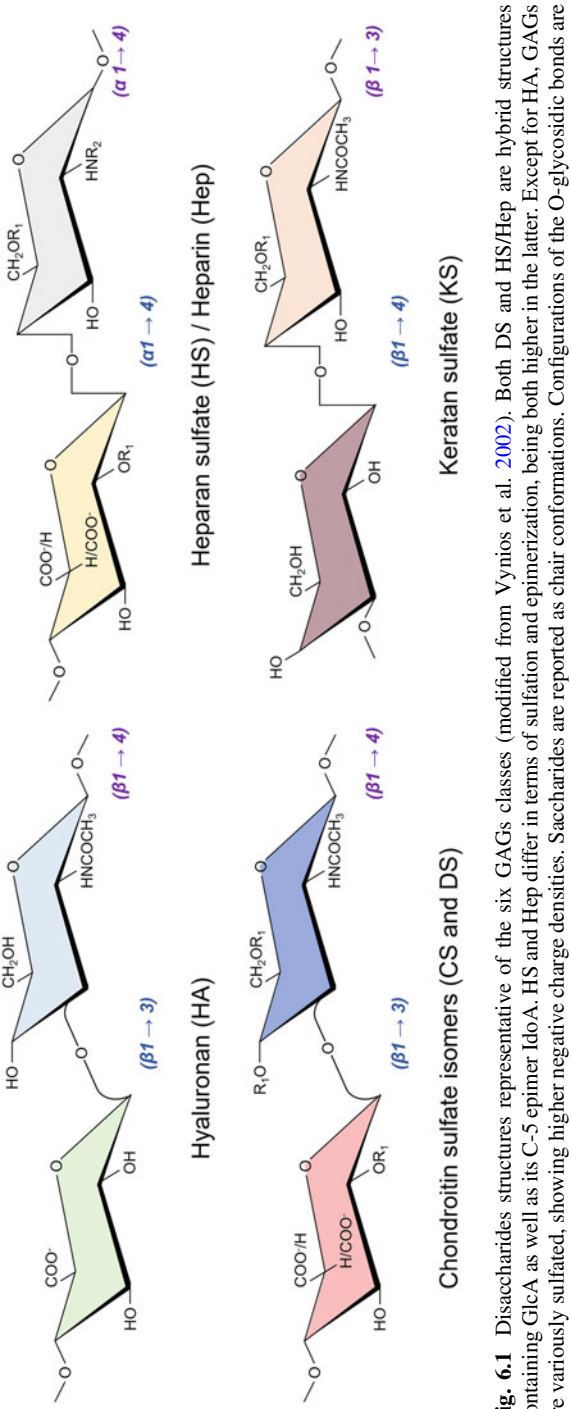

Fig. 6.1 Disaccharides structures representative of the six GAGs classes (modified from Vynios et al. 2002). Both DS and HS/Hep are hybrid structures containing GlcA as well as its C-5 epimer IdoA. HS and Hep differ in terms of sulfation and epimerization, being both higher in the latter. Except for HA, GAGs are variously sulfated, showing higher negative charge densities. Saccharides are reported as chair conformations. Configurations of the O-glycosidic bonds are reported either in blue (within disaccharide units) or in purple (between adjacent disaccharide units). $R_1 = SO_3^-$; $R_2 = COCH_3/SO_3^-$

subfamily); the pericellular PGs are perlecan, agrin, collagens XV and XVIII; those connected to the cell surface comprise glypicans (GPCs) (belonging to the GPI-anchored PGs subfamily), syndecans (SDCs) CSPG4, betaglycan, phosphacan, and CD44 (belonging to the transmembrane PGs subfamily), whereas the only known intracellular PG is serglycin (Fig. 6.2) (Theocharis et al. 2010; Karamanos et al. 2021).

Regarding their function, SDCs and GPCs play multiple crucial roles in both physiological and pathological conditions, ranging from regulation of cell–matrix interactions to cell signaling, when anchored to the cell as well as when in the free form as a result of shedding.

The whole ECM undergoes substantial remodeling during tumor formation and growth by the action of both proteolytic and glycosidic enzymes secreted in the tumor stroma by inflammatory cells. As a consequence of these events, either ECM PGs, cell surface PGs, or their degradation products, may be released into circulation, representing potential markers with diagnostic, prognostic, or therapeutic significance. Their high blood levels may be either the result of an intense proteolytic activity on constitutively expressed PGs (e.g., those physiologically present in ECM or glycocalyx) or to a de novo expression in tumor stroma (e.g., the expression of the oncofetal GPC-3 by hepatocellular carcinoma).

Recently, we performed a literature investigation to evaluate the clinical utility of measuring circulating HS/HSPGs in pathophysiological conditions showing interesting results on several inflammation-based pathologies such as cardiovascular disease, diabetes, obesity and kidney disease, trauma, sepsis, but also multiple sclerosis, preeclampsia, pathologies requiring surgery, pulmonary disease, and others (Lepedda et al. 2021b).

Several evidences show that the soluble forms, originating from shedding, are detectable in blood and other body fluids and represent useful diagnostic and/or prognostic tools of various malignancies responsible for high mortality and disability including, among others, hepatocellular carcinoma (HCC) and multiple myeloma (MM).

This chapter reviews the major clinical literature on this topic and provides an overview of the main findings. The items for PubMed search included: ECM, circulating PGs/HSPGs, HA, HS, GAGs, SDCs, GPCs, glycocalyx/shedding, circulating cancer biomarkers. More than 300 articles were reviewed. Among them, about 150 clinical studies dealt with the association between circulating GAGs/PGs levels, mainly originated from glycocalyx degradation, and several cancer types. Noteworthy, in almost all of them, the HSPGs SDCs, GPCs or their ectodomains from the cellular glycocalyx degradation, were chosen as biomarkers.

For more detailed discussion, readers are referred to excellent reviews on topics such as ECM structure (Theocharis et al. 2019; Karamanos et al. 2021), PGs structure and function (Theocharis et al. 2010; Theocharis and Karamanos 2019; Karamanos et al. 2021; Zolota et al. 2021), as well as endothelial glycocalyx structure and function (Weinbaum et al. 2007; Zhang et al. 2018b), in both physiological and pathological conditions.

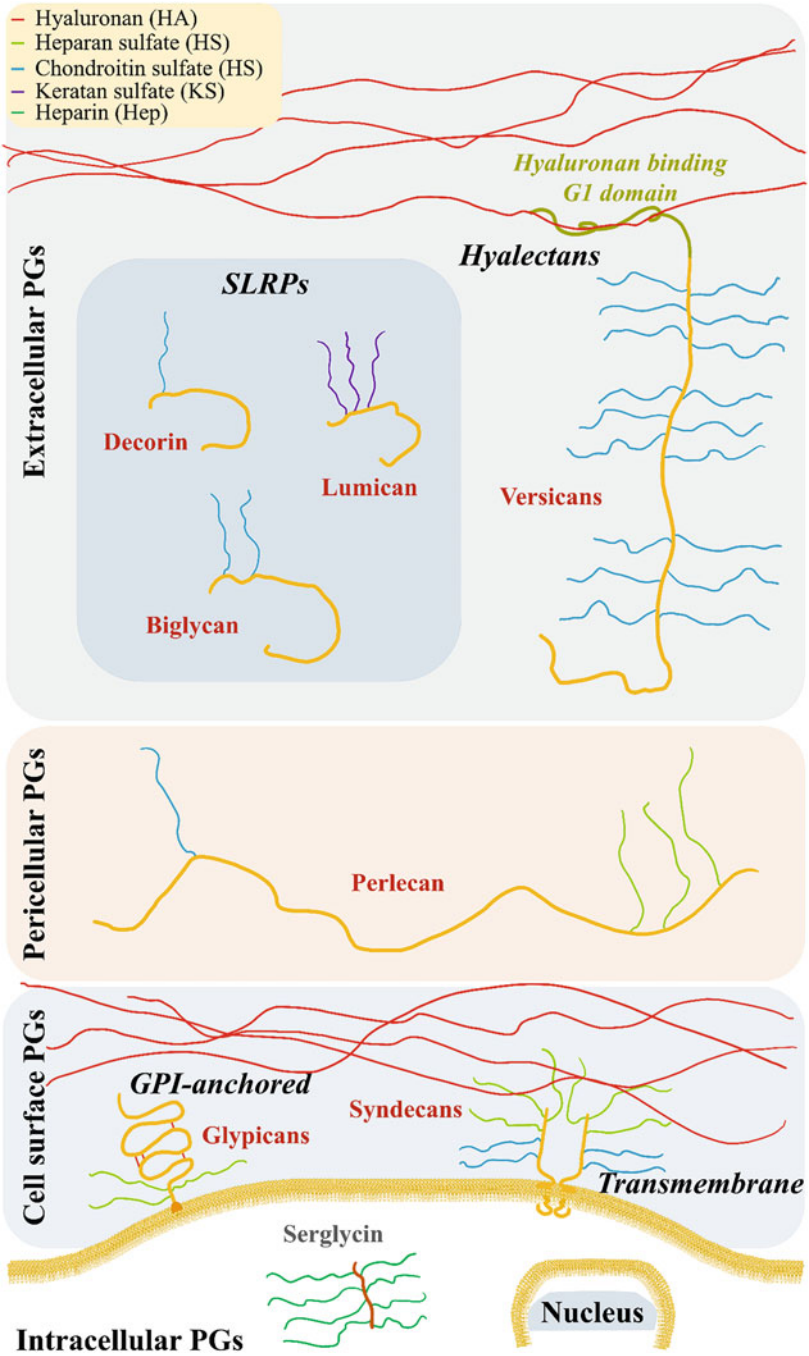

Fig. 6.2 Schematic representation of the four classes of PGs according to their localization: intracellular, cell surface, pericellular, and extracellular. Some representative PGs and the corresponding subfamilies are reported. Protein moieties are shown as yellow backbones (versican G1 domain is highlighted in green)

6.2 The Cellular Glycocalyx and Its Remodeling in Health and Disease

Every mammalian cell is covered by a dynamic and complex gel-like network, consisting of PGs (mainly SDCs and GPCs), glycoproteins (selectins, integrins, and immunoglobulins), glycolipids, and GAGs (HA, HS, and CS), collectively termed "glycocalyx," which is implicated in cell–cell and cell–matrix interactions.

Bennett et al. (1959) showed this structure, for the first time, on the luminal surface of vascular endothelial cells using electron microscopy (Bennett et al. 1959). Due to the high content of polysaccharides, particularly HS, CS isomers, and HA, it was called "glycocalyx" (literally meaning "sugar coat" from the ancient Greek words glykys/γλυκύς = sweet, kylix/κύλιξ = cup). The thickness (approximately 0.5–5.0μm) and structure of the glycocalyx vary in relation to the tissue, as well as with several pathophysiological conditions, and are the result of a dynamic balance between persistent biosynthesis and degradation, with a considerable rate of renewal of its components. PGs are major components of the cellular glycocalyx and participate to biochemical and mechanosignaling in cells. Changes in PGs expression in cancer and other pathologies have been associated with glycocalyx derangement and with intense effects on proliferation, adhesion, and migration (Barkovskaya et al. 2020). HS is covalently bound to core proteins to form the main glycocalyx PGs, SDCs, and GPCs (approximately 50–90% of the total amount of PGs), whereas HA binds noncovalently membrane proteins, such as CD44, providing structural support to the network. The cellular glycocalyx interacts with several matrix components, enzymes, proteins of plasma origin, pro-inflammatory cytokines, and growth factors, regulating cell–cell and cell–matrix crosstalk for maintaining tissue homeostasis (Zhang et al. 2018b). Derangement of the cellular glycocalyx structure, by either physical or chemical insults, plays essential roles in several pathological conditions, as reviewed with regard to pathologies such as cardiovascular disease (Machin et al. 2019), diabetes (Dogne et al. 2018), kidney disease (Jourde-Chiche et al. 2019), sepsis (Iba and Levy 2019; Uchimido et al. 2019), and trauma (Tuma et al. 2016). In particular, glycocalyx shedding, a regulated proteolytic cleavage of some of its components (Becker et al. 2015), may expose, in pathological conditions, adhesion molecules such as selectins and intercellular adhesion molecule 1, on the endothelium surface, thus inducing leukocytes and platelets recruitment, therefore leading to vascular dysfunction (Celie et al. 2009; Kumar et al. 2015). Furthermore, all these events increase plasma concentration of several glycocalyx components, such as HA, HS, and their degradation products, SDCs and GPCs ectodomains, which may in turn affect both local and systemic signaling pathways (Yang et al. 2017), in some cases lasting even for days as reported for highly sulfated HS fragments released in circulation in patients with respiratory failure (Schmidt et al. 2014).

Under various acute and chronic clinical conditions, the glycocalyx shedding primarily involves various MMPs and A disintegrin metalloproteinases with thrombospondin motifs (ADAMTS) -1 and -4, as well as heparanase and

hyaluronidase, released by inflammatory cells and activated endothelial cells, reactive oxygen and nitrogen species (ROS and RNS). Also serine proteases such as thrombin, elastase, proteinase 3, plasminogen, and cathepsin B are involved in glycocalyx shedding (Becker et al. 2015).

MMPs (MT1-MMP, MMP-2, -7, and -9) and heparanase are secreted following inflammatory stimuli and contribute to the cleavage of matrix components as well as of cell surface PGs. Heparanase cleaves HS chains, mainly at low sulfation sites, therefore releasing fragments of 4–7 kDa (Meirovitz et al. 2013). Besides the action of heparanase and other specific endoglycosidases, also ROS and RNS can lead to GAGs fragmentation (Moseley et al. 1997; Soltes et al. 2006) with fragments which can be released in circulation, representing potential useful markers of disease.

MMPs activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) which can bind HS and CS on the glycocalyx, whereas Hep is an inhibitor of heparanase (Oduah et al. 2016). In this respect, pharmaceutical mixtures of low-molecular weight Hep and CS isomers, such as sulodexide, can be used as antithrombotic and pro-fibrinolytic drugs with anti-inflammatory, antioxidant, and vasculoprotective properties, probably preserving endothelial glycocalyx from shedding by heparanase and MMP-9 activities (Weiss et al. 2007).

HA is a ubiquitous ECM and glycocalyx component that plays key roles during embryogenesis, inflammation, wound healing, and, particularly, in neoplasia where it modulates the crosstalk between cancer cells and the host peritumor stroma (Stern 2008; Passi et al. 2019; Caon et al. 2020). It is a linear, high-molecular weight polymer (up to 3–4 MDa) composed of long unbranched and nonsulfated free chains (1–2μm) of repeating GlcA and GlcNAc residues connected by β-1,3- and β-1,4-glycosidic bonds (Passi et al. 2019). Unlike the other GAGs, which are synthesized in the Golgi apparatus bound to a protein moiety, HA is synthesized by membrane-bound synthases at the inner side of the plasma membrane and then it is extruded through pore-like structures to the cell surface without undergoing any further postsynthetic modifications (i.e., sulfation or epimerization) (Vigetti et al. 2014). HA interacts with cell surface by specific receptors, such as the transmembrane CD44 and the receptor for HA-mediated motility (RHAMM), modulating cell behavior differently, according to its molecular weight (Tavianatou et al. 2019a; Caon et al. 2020). In particular, it is widely acknowledged that high-molecular weight HA has antiangiogenic, antiproliferative, and immunosuppressive properties, whereas low-molecular weight HA fragments are pro-inflammatory, pro-angiogenic, and could favor cancer cell aggressive phenotype (Tavianatou et al. 2019b, 2021). HA is synthesized at high levels in several tumors by the action of HA synthase-2, the most catalytically active isoform, strongly related to tumor aggressiveness and unfavorable outcome (Caon et al. 2020). Moreover, its degradation by the action of hyaluronidase 1 (the most active somatic hyaluronidase), and hyaluronidase 2 (a glycosylphosphatidylinositol (GPI)-anchored enzyme) during extracellular remodeling in tumorigenesis is increased and causes the release of bioactive fragments. Accordingly, both high- and low-molecular weight HA have been found at high levels in plasma, particularly in breast cancer patients (Velesiotis et al. 2019), where they have been associated with metastasis onset and poor prognosis, and used

as useful markers for monitoring the response to therapy (Wu et al. 2015; Peng et al. 2016).

Besides changes in HA metabolism, also the biosynthetic pathways of the other GAGs may undergo consistent alterations in cancer, as elegantly shown in clear cell renal cell carcinoma (ccRCC), resulting in altered metabolite levels in plasma. Indeed, Gatto et al. (2016b), using genome-scale metabolic modeling, evidenced deep changes about GAGs biosynthesis in ccRCC and identified distinct GAGs profiles in plasma and urine of patients. They calculated scores based on 18 different GAGs properties, including CS and HS levels, disaccharides composition, and charge density, with diagnostic accuracy ranging from 93.1 to 100% (Gatto et al. 2016b) and high prognostic value (Gatto et al. 2016a), also in surgically treated patients, independently from stage, grade, or histology (Gatto et al. 2018).

6.2.1 Cell Surface PGs

Type I transmembrane SDCs, together with glycosyl-phosphatidylinositol-anchored GPCs, represent the two major families of cell surface PGs. They are structural glycocalyx components that play major roles in regulating cell behavior, cell signaling, and cell–matrix interactions.

SDCs are a well-conserved family of single-span transmembrane HSPGs (both SDC-1 and -3 bear also CS chains) with pleiotropic roles in development, inflammation, and tumor progression by modulating cell proliferation, differentiation, adhesion, and migration (Chung et al. 2016; Afratis et al. 2017). Their core proteins, ranging from 20 to 40 kDa (33, 23, 41, and 22 kDa for SDC-1, -2, -3, and -4, respectively), are coded by four genes in vertebrates with distinct spatio-temporal pattern of expression (Gondelaud and Ricard-Blum 2019). Accordingly, SDC-1 is widely expressed in both epithelial and plasma cells, while SDC-2 is mainly expressed in mesenchymal cells, such as fibroblasts and smooth muscle cells, SDC-3 is expressed in neural tissues and developing musculoskeletal tissues, whereas SDC-4 is abundant in most tissues (Afratis et al. 2017). Their expression is finely regulated, since SDCs are involved in several signaling events, including tumorigenesis. Each isoform consists of a short C-terminal cytoplasmic domain, a transmembrane domain and an ectodomain carrying three to five HS or CS chains (Fig. 6.2). A model of full-length SDC-3 has been generated though scanty structural data are currently available, probably because of the high percentages of disordered amino acid residues (Peysselon et al. 2011). Following alternative splicing removal of the transmembrane domain, SDC-4 may occur also as a soluble protein isoform (Xing et al. 2003). The short cytoplasmic domain, consisting of the two conserved C1 and C2 regions, located respectively near the transmembrane domain and at the C-terminal, and a variable V region in between specific to each SDC and highly conserved across different species (Fig. 6.2), interacts with several intracellular kinases, as well as with actin cytoskeleton, thus modulating signal transduction (Couchman et al. 2015). Both C1 and C2 domains are highly conserved throughout

the animal kingdom (Chakravarti and Adams 2006), being C1 identical in all four mammalian SDCs. This latter participates in SDC di- or oligomerization, as well as in binding of several intracellular proteins. In SDC-4, this region includes a phosphatidylinositol-4,5-bisphosphate (PIP_2) binding site implicated in dimerization and required for protein kinase C activation and intracellular signaling (Horowitz et al. 1999, 2002; Murakami et al. 2002). C2 domain has a well-conserved carboxyl-terminal EFYA tetrapeptide sequence able to bind some PDZ domain-containing proteins that may have a role as scaffold proteins for the recruitment of signaling and cytoskeletal proteins to the plasma membrane (Cheng et al. 2016). Besides, it is also implicated in intracellular transduction by organizing signaling complexes at cell membrane. The transmembrane domain is highly conserved and contains a GXXXG motif that promotes self-association, thus leading to the formation of dimers/oligomers essential for protein kinase C activation (Oh et al. 1997). The ectodomain, interacts with the extracellular milieu primarily by its GAGs chains and is fundamental for the mechanisms of signal transduction (Chu et al. 2004). Its shedding is mainly carried out by MMPs and occurs constitutively at specific sites (Becker et al. 2015), but it is fairly accelerated consequently to both inflammatory stimuli and pathological conditions (Schmidt et al. 2005; Piperigkou et al. 2016; Zhang et al. 2018b). This leads to a rapid modulation of intracellular response, by reducing surface receptors as well as generating soluble bioactive ectodomains. HS chains are covalently O-linked to serine residues in a serine-glycine motif surrounded by acidic residues near the N-terminal of ectodomain. Furthermore, SDC-1 and -3 carry CS/DS chains at sites closer to the transmembrane domain. Polymerization of HS and CS/DS chains occurs in the Golgi apparatus, starting from a xylose–galactose–galactose–uronic acid tetrasaccharide linker, and it is carried out by the sequential and repetitive addition of GlcA and either GlcNAc (HS chain) or GalNAc (CS chain) residues, to reach 50–200 disaccharides in length. During polymerization, both iduronation and sulfation at specific positions occur, mediated by glucuronyl C-5 epimerase and by sulfotransferases, respectively, hence leading to extensive chain modification. With regard to HS, sulfation may occur at the 2-O position of the hexuronic acid residues, whereas N-glucosamine residues may be N-sulfated, sulfated at 6-O position or, less frequently, at the 3-O position. The different disaccharides species are not randomly distributed but produce alternating subdomains of low (N-acetylated domains) and high sulfation (N-sulfated domains) with intermediate sulfation sequences in between (N-sulfated/N-acetylated domains) (Fig. 6.3).

After being exposed at the cell surface, HS chains may be further modified by specific sulfatases, which selectively remove 6-O sulfates (Morimoto-Tomita et al. 2002; Bishop et al. 2007; Nadanaka and Kitagawa 2008). Due to their high structural heterogeneity, HS can interact with a wide range of ligands such as ECM proteins (e.g., collagens, fibronectin), plasma proteins (e.g., albumin, antithrombin), cytokines, chemokines, growth factors (e.g., FGF, VEGF, TGF-β, and platelet-derived growth factor (PDGF)), and enzymes, which are primarily involved in signal transduction by forming ternary complexes with both growth factors and their receptors, e.g., with FGF2-FGFR1 (Qiao et al. 2003; Tkachenko et al. 2005). During glycocalyx shedding, the endo-β-D-glucuronidase heparanase may cleave HS chains

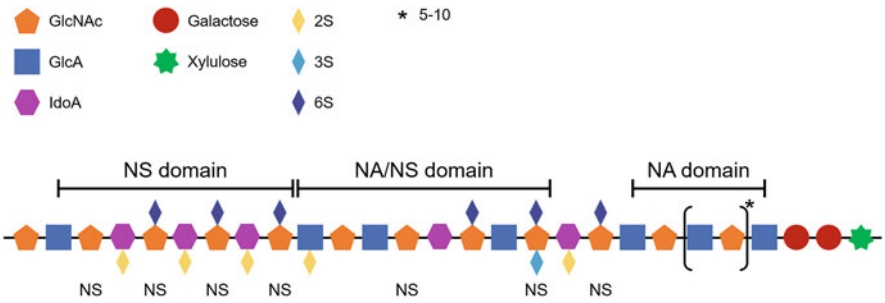

Fig. 6.3 Repetitive domain organization of HS. Highly sulfated/iduronated regions (N-sulfated domains or NS domains) are flanked by moderately modified regions (N-acetylated/N-sulfated domains or NA/NS domains) with interspersed unmodified sequences (N-acetylated domains or NA domains) (Esko and Selleck 2002)

at sites of low-sulfation, with the release of 10–20 units-long oligosaccharides, thereby converting HS from an inhibitor to a potent activator of FGF-2 (Kato et al. 1998; Barash et al. 2010). So far, little is known about the functions of SDCs CS chains, for which has been suggested a role in modulating cell adhesion to the ECM, together with HS chains (Okamoto et al. 2003). Recently, Gondelaud and Ricard-Blum have provided a comprehensive SDC interactome consisting of 351 partners, identified also by high-throughput affinity purification-mass spectrometry (Gondelaud and Ricard-Blum 2019). Very interestingly, it has been shown that most of these interactions are mediated by the GAGs moiety (71% for SDC-1, 67% for SDC-2, 91% for SDC-3, and 68% for SDC-4).

GPCs are a family of six (in mammals) GPI-anchored HSPGs (GPC-1 to -6), organized into two subfamilies (GPCs -1, -2, -4, -6 and GPCs -3 and -5). They have core proteins ranging from 60 to 70 kDa with about 25% amino-acid identity with each other, and very similar three-dimensional structures stabilized by 7 disulfide bridges, originated by 14 well-conserved cysteine residues distributed in a large globular cysteine-rich domain (Filmus and Selleck 2001; Filmus et al. 2008). An hydrophobic C-terminal domain, required for the binding of the GPI anchor, links GPCs to specific plasma membrane lipid rafts enriched in cholesterol and sphingolipids, and is involved in vesicular transport and cell signaling (Belting 2003). The last 50 amino acids sequence in the C-terminus is close to the membrane and contains attachment sites for 3–4 HS chains. During maturation in the secretory pathway, the endoprotease Furin may cleave GPCs (between Arg^{358} and Ser^{359} in GPC-3) in 2 fragments of 40 and 30 kDa (the N-terminal and the C-terminal subunits, respectively) connected by disulfide bridges (De Cat et al. 2003; Hippo et al. 2004). GPCs can also be found in circulation either as full-length glycated form following cleavage by Notum, a kind of lipase that cleaves GPI-anchored proteins (Traister et al. 2008), or as N-terminal fragments lacking HS side chains (Hippo et al. 2004). GPCs are usually expressed at high levels during embryonic development with tissue-specific and stage-specific expression, suggesting a fundamental role in various biological processes, such as interaction between cell and ECM, as well as

the control of cell division, differentiation, and morphogenesis (Filmus and Selleck 2001). GPC-1 is expressed in bone, bone marrow, muscle, epithelium, and kidney; GPC-2 is specifically present in nervous system; GPC-3 and GPC-6 are produced in most tissues; GPC-4 is produced in brain, kidney, and lung, whereas GPC-5 is expressed in brain, lung, liver, kidney, and limbs. In adults, significant alterations in the pattern of GPCs expression have been reported. In particular, GPC-1, GPC-4, and GPC-6 are widely expressed in various tissues, while GPC-2 is no longer expressed; GPC-3 is reported only in the ovary, mammary gland, mesothelium, lung, and kidney, whereas GPC-5 is specifically found in the brain (Filmus 2001). However, among all GPCs, only GPC-1 is expressed by endothelial cells (Rosenberg et al. 1997). GPCs are involved in the regulation of pathways including Wnt, FGF, Hedgehog, bone morphogenic protein, Slit, and insulin-like growth factor, with either stimulatory or inhibitory activity depending on the biological context (Filmus et al. 2008; Theocharis et al. 2010).

6.3 Circulating PGs/GAGs as Cancer Biomarkers

As mentioned above, ECM is tightly involved in tissue homeostasis as well as in the pathogenesis of several diseases such as cancer and undergoes deep remodeling by the activities of both proteolytic and glycosidic enzymes secreted by inflammatory cells. Besides, also the synthesis rate and the expression pattern of some of its components may vary greatly.

PGs play key roles in tumorigenesis, from regulation of cell–matrix interactions to cell signaling. Their soluble forms, detectable in plasma or other fluids, may be useful diagnostic and/or prognostic tools for several malignancies, overall responsible for about 14% of global deaths and 7.5% of total disability-adjusted life year (DALY). This parameter shows severity of a given disease by combining years of life lost due to premature mortality and disability (https://www.who.int/data/gho/indicator-metadata-registry/imr-details/158). A picture of the burden of these diseases is shown in Table 6.1 where percentages of global deaths and total DALY for each cancer type are reported.

The following discussion is focused on HCC and MM, two cancers responsible for high mortality and disability. In these cancers, the cell surface HSPGs GPC-3 and SDC-1 are well-established markers, whereas the main findings on the significance of circulating PGs/GAGs in prognosis and diagnosis of other tumor types are summarized in Table 6.2.

6.3.1 Hepatocellular Carcinoma

According to the International Agency for Research on Cancer, liver cancer is the third most common cause of cancer death leading to 781,631 deaths worldwide

Table 6.1 List of cancer types in which a significant relationship with plasma GAGs/PGs levels has been reported, in the last 20 years (https://pubmed.ncbi.nlm.nih.gov). Percentages of global deaths and of total disability-adjusted life years (DALYs) for 2019 are shown

Cancer type	% of global deaths	% of total DALY
Liver cancer	0.86	0.49
Multiple myeloma	0.20	0.099
Prostate cancer	0.86	0.34
Pancreatic cancer	0.94	0.46
Larynx cancer	0.22	0.13
Esophageal cancer	0.88	0.46
Stomach cancer	1.69	0.88
Breast cancer	1.24	0.81
Kidney cancer	0.29	0.16
Colon and rectum cancer	1.92	0.96
Tracheal, bronchus, and lung cancer	3.61	1.81
Leukemia	0.59	0.46
Ovarian cancer	0.35	0.21
Hodgkin's lymphoma	0.05	0.045
Malignant skin melanoma	0.11	0.067
Bladder cancer	0.40	0.17
Total	14.21	7.55

Source: https://vizhub.healthdata.org/gbd-compare/

(8.2%) (Source: Bray et al. 2018), being HCC the major contributor. Chronic persistent infections of hepatitis B or C virus are primary risk factors for HCC, but an association with environmental factors, such as alcohol consumption or exposure to some chemicals and aflatoxin B1 has been reported as well. HCC is a very aggressive cancer characterized by a considerably low 5-year survival rates (El-Serag 2011).

As demonstrated in 1997 by Hsu et al., GPC-3 mRNA is highly expressed in embryonal tissues such as placenta, liver, lung, and kidney, whereas it is undetectable (e.g., liver) or barely detectable (e.g., lung and kidney) in almost all adult tissues. Furthermore, Hsu et al. (1997) showed that it is highly and specifically expressed in HCC (Hsu et al. 1997). These findings were followed by many studies indicating GPC-3 as a reliable immunohistochemical diagnostic marker of prognostic significance in predicting overall survival and disease-free survival in HCC patients (Table 6.3). Indeed, a GPC-3 expression has been associated with tumor stage, differentiation, and presence of vascular invasion and metastasis (Li et al. 2014; Xiao et al. 2014; Liu et al. 2018; Zhang et al. 2018a; Moudi et al. 2019). Interestingly, as it is highly and specifically expressed on HCC cells' surface, it could also represent a promising target for immunotherapies, treatment with peptide/DNA vaccines, immunotoxin, or genetic therapies (Zhou et al. 2018b; Guo et al. 2020).

As mentioned above, during maturation, the endoprotease Furin can cleave GPC-3 between Arg^{358} and Ser^{359} residues, thus generating 2 fragments of 40 and

Table 6.2 Main findings of studies correlating circulating PGs/GAGs levels with tumors other than HCC or MM

References	Tumor typology	PGs/GAGs	Casuistry	Main findings
Grindel et al. (2016)	Prostate Cancer (PC)	Perlecan	288 PC vs. 12 healthy controls	Perlecan fragments associated with MMP-7 in prostate cancer tissues; domain IV perlecan in stage IV, but absent in normal sera. Perlecan fragments in sera and MMP-7 in tissues are measures of invasive prostate cancer
Szarvas et al. (2016)		SDC-1	99 PC	SDC-1 levels as promising tool for pre-operative risk-stratification and/or therapy monitoring
Arslan et al. (2017)		Endocan	86 PC vs. 80 controls	High serum endocan levels ($\geq$1.8 ng/ml) is a significant predictor of biochemical progression-free survival (hazard ratio 2.44; 95% confidence interval 1.78–3.23; $p = 0.001$)
da Silva et al. (2018)		HA	44 PC vs. 14 controls	39.68 ± 30.00 ng/ml vs. 15.04 ± 7.11 ng/ml ($p < 0.004$); potentially useful biomarker for the diagnosis and prognosis of prostate cancer
Levin et al. (2018)		GPC-1	15 PC vs. 15 benign prostatic hyperplasia vs. controls	GPC-1 reduction in prostate cancer patients ($p < 0.05$)
Szarvas et al. (2018)		SDC-1	75 PC who received docetaxel therapy until the appearance of therapy resistance	Serum SDC-1 may help to facilitate clinical decision-making regarding the type and timing of therapy for patients with castration-resistant prostate cancer (CRPC); positive correlation between SDC-1 and MMP7

(continued)

Table 6.2 (continued)

References	Tumor typology	PGs/GAGs	Casuistry	Main findings
Melo et al. (2015)	Pancreatic Ductal Adenocarcinoma (PDAC)	GPC-1^{+} circulating exosomes (crExos)	190 PDAC vs. 32 breast cancer vs. 100 healthy donors	GPC-1^{+} crExos potential marker for detection of early stages of pancreatic cancer; correlation with tumor burden and the survival of pre- and post-surgical patients
Lai et al. (2017)		Exosomal GPC-1	3 PDAC vs. 3 chronic pancreatitis vs. 6 healthy controls	GPC-1 is not diagnostic for Pancreatic ductal adenocarcinoma
Frampton et al. (2018)		GPC-1^{+} circulating exosomes (crExos)	27 PDAC vs. 16 benign pancreatic disease	GPC-1 levels enriched in PDAC crExos; GPC-1 association with PDAC tumor size and disease burden
Zhou et al. (2018a)		GPC-1	156 PDAC vs. 199 non-cancer controls vs. 240 patients with other cancers	High GPC-1 levels association with poor prognosis in PDAC
Chen et al. (2020)		HA	809 PDAC vs. 44 ampullary carcinoma vs. 31 distal biliary tract cancer vs. 15 chronic pancreatitis vs. 41 intraductal papillarymucinous neoplasm vs. 7 duodenal adenoma vs. 25 no cancer	Higher baseline serum HA in pancreatic cancer than healthy subjects and patients with benign conditions; association with overall survival
Xiao et al. (2020)		Exosomal GPC-1 (panel of exosomal proteins)	24 pancreatic cancer vs. 6 chronic pancreatitis vs. 23 acute pancreatitis vs. 9 benign breast tumor vs. 11 breast cancer vs. 26 Healthy people	Specific, sensitive, and reproducible detection panel for the diagnosis of pancreatic cancer

Anttonen et al. (2006)	Upper gastrointestinal cancers	SDC-1	44 larynx and hypopharynx carcinomas	Higher SDC-1 levels correlate with cancer recurrence
Wu et al. (2010)		Decorin	275 esophageal squamous cell carcinoma vs. 295 controls	Decorin levels significantly lower in patients than in controls ($p < 0.0001$)
Ruan et al. (2011)		HA	50 gastric cancer vs. 41 precancerous lesion vs. 30 control subjects	Higher HA levels in patients with gastric cancer than in patients with precancerous lesion and control group ($p < 0.05$, $p < 0.0001$)
Aghcheli et al. (2012)		HA	20 gastric cardia cancer vs. 23 gastric noncardia cancer vs. 20 esophageal squamous cell carcinoma incident cases vs. 25 controls	Higher HA levels in cancer ($p < 0.01$); useful to identify potentially high-risk groups of upper gastrointestinal cancers
Shegefti et al. (2016)		SDC-1	43 Tongue squamous cell carcinoma (SCC) vs. 46 controls	Lower levels of SDC-1 in patients (91.17 ± 88.60 vs. 158.17 ± 103.47 ng/ml, $p = 0.002$); no correlation with the tumor progression in the tongue SCC
Li et al. (2017)	Colorectal Cancer (CRC)	Exosomal GPC-1	102 CRC vs. 80 healthy controls	Plasma GPC-1+ exosomes, miR-96-5p and miR-149 are specific markers for the diagnosis of CRC and targets for the therapy
Wang et al. (2014a)		SDC-1	52 resectable primary CRC vs. 11 controls	High SDC-1 levels correlated with chemotherapy resistance; potential new prognostic marker in colorectal cancer
Jary et al. (2016)		SDC-1	Development cohort: 126 metastatic CRC, validation cohort: 51 patients	SDC-1 is an independent prognostic factor in metastatic CRC patients
Wu et al. (2019)		~6 kDa HA	184 CRC vs. 75 benign diseases vs. 63 controls	Potential biomarker for diagnosing CRC and predicting early relapse, cancer progression, and lymph node metastasis; prompt decrease after tumor removal

(continued)

Table 6.2 (continued)

References	Tumor typology	PGs/GAGs	Casuistry	Main findings
Joensuu et al. (2002)	Lung cancer (LC)	SDC-1	138 nonsmall cell LC vs. 46 small cell LC	Survival: SDC-1 > 59 ng/ml vs. SDC-1 < 59 ng/ml, 4 months vs. 11 months ($p < 0.0001$); high serum SDC-1 levels at diagnosis are associated with poor outcome, independently from the histological subtype
Anttonen et al. (2003)		SDC-1	88 small cell LC	High pre-treatment serum SDC-1 level association with poor prognosis in patients treated with platinum-based chemotherapy
Nishio et al. (2011)		HS	83 stage IV nonsmall cell LC	Higher HS levels are significantly associated with shorter progression-free survival, and overall survival ($p = 0.0012$ and $p = 0.0003$)
Jilani et al. (2009)	Chronic lymphocytic leukemia (CLL)	SDC-1	104 CLL vs. 32 controls	52.8 ng/ml (13.4–252.7 ng/ml) vs. 19.86 ng/ml (14.49–33.14 ng/ml) ($p < 0.01$)
Wolowiec et al. (2006)		SDC-1	52 CLL vs. 12 healthy controls	Higher SDC-1 levels in early-stage B-CLL patients as well as in patients with more indolent disease course than in healthy controls
Molica et al. (2006)		SDC-1	67 CLL (Binet stage A, 46; stage B, 7; stage C, 14) vs. 15 healthy controls	Lower levels in patients than in controls ($p = 0.02$); no correlation with clinical stages
Wolowiec et al. (2006)		SDC-1	52 CLL vs. 12 healthy controls	149.0 ng/ml (13.2–257.1 ng/ml) vs. 36.7 ng/ml (17.4–135.8 ng/ml) ($p = 0.0002$)

Larsen et al. (2013)	Acute myeloid leukemia (AML)	SDC-1	49 AML	High levels of SDC-1 associated with bleeding, impaired platelet function, higher age, endothelial cell activation and damage, and leukocytosis
Anagnostopoulou et al. (2017)		HA	51 AML, B-acute lymphoblastic leukemia (BALL), and mantle-cell lymphoma (MCL) vs. 52 controls	High HA levels in patients with AML, B-ALL, and MCL; inverse correlation between overall survival and HA levels
Alghandour et al. (2020)		SDC-1	24 AML vs. 16 acute lymphoblastic leukemia vs. 15 healthy subjects	Useful diagnostic and prognostic marker for AML but not for acute lymphoblastic leukemia
Thylen et al. (1999)	Malignant pleural mesothelioma	HA	19 Malignant pleural mesotheliomas	Positive correlation between plasma and pleural fluid levels of HA. Positive correlation between HA levels and tumor volume in HA-producing group of mesotheliomas ($p = 0.01$)
Burchardt et al. (2003)	Melanoma	HA	6 malignant melanomas (stage I/II and 6 stage IV)	Significant increase in all stages of disease
Ikuta et al. (2005)		GPC-3	109 melanoma vs. 5 large congenital melanocytic nevus vs. 61 age-matched healthy donors vs. 13 disease-free patients after undergoing a surgical removal	Potential marker of melanoma, especially at an early stage
Vassilakopoulos et al. (2005)	Hodgkin's lymphoma	SDC-1	66 Hodgkin's lymphoma vs. 14 healthy controls	100.2 ± 35.9 ng/ml vs. 67.9 ± 24.5 ng/ml ($p < 0.001$)
Pothacharoen et al. (2006)	Ovarian cancer	CS and HA	91 ovarian epithelial cancer vs. 39 noncancer gynecological disorders vs. 30 healthy women	CS levels highly increased in epithelial types of ovarian cancer and at all stages of development ($p < 0.005$); HA levels significantly higher in ovarian cancer patients than in normal controls ($p < 0.05$)
Obayashi et al. (2008)		HA, SHAP-HA complex, UTI	45 ovarian cancer vs. 22 benign ovarian tumors vs. 50 healthy women	Levels of HA and SHAP-HA complex higher in ovarian cancer; elevated SHAP-HA complex associated with shorter disease-free survival

(continued)

Table 6.2 (continued)

References	Tumor typology	PGs/GAGs	Casuistry	Main findings
Zhou et al. (2017)	Hepatoblastoma (HB)	GPC-3	134 HB vs. 30 benign hepatobiliary disorders vs. 20 controls	1.93 ng/ml (0–31.19) vs. 1.74 ng/ml (0–25.95) ($p = 0.6$) vs. 0.59 ng/ml (0–6.20) ($p = 0.003$); GPC-3 is inferior to AFP as a serum marker for HB
Appunni et al. (2017)	Bladder cancer	Decorin, Biglycan and Lumican	30 bladder cancer vs. 30 healthy controls	Higher plasma levels of Biglycan ($p = 0.0038$) and Lumican ($p < 0.0001$) and reduced levels of Decorin ($p < 0.0001$) in urothelial carcinoma of bladder
Indira Chandran et al. (2019)	Glioblastoma	SDC-1 extracellular vesicles	136 high-grade glioblastoma multiforme and low-grade glioma	Potential tool for minimally invasive diagnosis of glioma

Table 6.3 Meta-analysis studies on the prognostic significance of high hepatic tissue GPC-3 expression in predicting overall survival (OS) and disease-free survival (DFS) of HCC patients

References	Casuistry	Assay	Overall survival[a]	Disease-free survival[b]
Li et al. (2014)	493 patients/5 studies	IHC	HR = 2.18, 95% CI: 1.47–3.24, $p = 0.0001$	HR = 2.05, 95% CI: 1.43–2.93, $p < 0.0001$
Xiao et al. (2014)	1070 patients/ 8 studies	IHC	HR = 1.96, 95% CI: 1.51–2.55, $p = 0.000$	HR = 1.99, 95% CI: 1.57–2.51, $p = 0.000$
Liu et al. (2018)	2336 patients/ 15 studies	IHC	HR = 1.38 95% CI: 1.05–1.80, $p = 0.02$	HR = 1.98, 95% CI: 1.08–3.62, $p = 0.027$
Zhang et al. (2018a)	2364 patients/ 14 studies	IHC	HR = 1.40, 95% CI: 1.07–1.85, $p = 0.02$	HR = 1.61, 95% CI: 1.13–2.30, $p = 0.008$
Moudi et al. (2019)	2618 patients/ 17 studies	IHC	HR = 1.57, 95% CI: 1.18–2.10, $p = 0.002$	HR = 1.93, 95% CI: 1.09–3.43, $p = 0.02$

IHC immunohistochemistry, *HR* hazard ratio, *CI* confidence interval
[a]Association between overexpression of GPC-3 and decreased overall survival
[b]Association between overexpression of GPC-3 and decreased disease-free survival

a 30 kDa, respectively, the NH_2-terminal and the COOH-terminal, which carries the sites for HS chains attachment, linked together by disulfide bonds (De Cat et al. 2003; Hippo et al. 2004). The former can be released in serum of HCC patients, representing an effective circulating marker for early-stage HCC (Hippo et al. 2004).

Taking its aggressiveness and the associated poor prognosis into account, identification of reliable early markers is mandatory. With this aim, several studies have focused on validating serum GPC-3 as a noninvasive diagnostic marker of HCC (Table 6.4), most of which have been included in the six meta-analysis studies reported in Table 6.5 (Xu et al. 2013, 2019; Jia et al. 2014; Liu et al. 2014, 2015; Yang et al. 2014).

These studies calculated pooled accuracy parameters showing a wide range of sensitivity (from 53 to 69%), specificity (from 58 to 93%), and diagnostic odds ratio (from 3.6 to 31), thus suggesting serum GPC-3 as a promising diagnostic marker for HCC. However, the great heterogeneity among studies (I^2 values were considerably higher than the cut-off value of 50%, as reported in Table 6.5) and major design deficiencies, including lack of clear exclusion and inclusion criteria, have therefore led to nondefinitive conclusions.

To improve the diagnostic power of GPC-3 for HCC, Attallah et al. (2016) developed a GPC–HCC model based on the combination of GPC-3 levels and routine laboratory tests, including the α-fetoprotein actually considered the gold standard tumor marker for HCC, alanine transaminase, aspartate transaminase, alkaline phosphatase, total bilirubin, albumin, and platelet count (Attallah et al. 2016). A cohort of 138 HCC patients, 56 with cirrhosis *versus* 62 with liver fibrosis were assessed, showing high diagnostic power with 93% sensitivity, 93% specificity, 89% positive predictive value, 95% negative predictive value, and 93% efficiency.

Despite being conducted on a smaller cohort of patients, namely 39 HCC patients *versus* 62 affected by liver fibrosis, also Jing et al. (2017) obtained high diagnostic

Table 6.4 Main findings of studies on the association of serum PGs/GAGs levels with HCC, carried out in the last 20 years

References	PGs/GAGs	Casuistry	Main findings
Nakatsura et al. (2003)	GPC-3	40 HCC vs. 13 liver cirrhosis vs. 34 chronic hepatitis vs. 60 healthy donors	GPC-3 positivity in serum: 40.0% in HCC vs. 0% in the other groups
Xu et al. (2013)	GPC-3	34 HCC vs. 20 hepatitis plus liver cirrhosis vs. 18 hepatitis vs. 53 controls	GPC-3 undetectable in both healthy individuals and patients with hepatitis; elevated GPC-3 in 53% of patients with HCC; elevated GPC-3 in 5% of patients with hepatitis plus cirrhosis
Nakatsura et al. (2003)	HA	28 chronic hepatitis vs. 43 liver cirrhosis vs. 57 HCC vs. 60 healthy controls	HA levels correlate with the extent of liver fibrosis and severity of cirrhosis
Hippo et al. (2004)	GPC-3	69 HCC vs. 38 liver cirrhosis vs. 96 healthy controls	4.84 ± 8.91 ng/ml vs. 1.09 ± 0.74 ng/ml ($p < 0.01$) vs. 0.65 ± 0.32 ng/ml ($p < 0.001$)
Zolota et al. (2021)	GPC-3	50 HCC with cirrhosis vs. 41 cirrhosis	161.41 ± 422.33 ng/ml vs. 125.41 ± 281.05 ng/ml (not significant)
Yasuda et al. (2010)	GPC-3	200 HCC vs. 200 chronic liver disease	924.8 pg/ml (495.2–1335.6 pg/ml) vs. 1161.6 pg/ml (762.0–1784.0 pg/ml) ($p < 0.0001$); lower expression in HCC
Tangkijvanich et al. (2010)	GPC-3	100 HCC vs. 50 intrahepatic cholangiocarcinoma vs. 50 metastatic carcinoma vs. 50 liver cirrhosis vs. 50 chronic hepatitis vs. 40 healthy controls	Elevated serum GPC-3 in 53% of HCC (ranging 35.5–7826.6 ng/ml) but undetectable in the other groups; Serum GPC-3 is highly specific for detecting HCC
Suzuki et al. (2010)	GPC-3	HCC vs. Chronic liver disease	800 ± 200 ng/ml vs. 350 ± 50 ng/ml ($p < 0.05$)
Liu et al. (2010)	GPC-3	37 HCC vs. 32 liver cirrhosis	GPC-3 is a sensitive, specific serum and tissue marker for the diagnosis of early HCC
Zhang et al. (2010)	GPC-3	36 HCC vs. 20 secondary liver cancer vs. 25 hepatitis B vs. 20 hepatitis C vs. 28 cirrhosis vs. 56 controls	116.8 ± 98.6 ng/ml vs. 24.60 ± 24.01 ng/ml vs. 13.67 ± 15.68 ng/ml vs. 6.73 ± 1.22 ng/ml vs. 0.86 ± 1.12 ng/ml, $p < 0.05$
Qiao et al. (2011)	GPC-3	101 HC vs. 40 cirrhosis vs. 18 hepatitis vs. 30 controls	GPC-3 is a useful tumor marker complementary to AFP for clinical diagnosis of HCC

Ozkan et al. (2011)	GPC-3	75 HCC vs. 55 cirrhosis vs. 28 healthy controls	5.13 pg/ml (53.9–93.2 pg/ml) vs. 5.51 pg/ml (53.9–236.2 pg/ml) vs. 3.9 pg/ml (53.9–7.7 pg/ml); Diagnostic values: Sensitivity 61.33%, Specificity 41.82%, positive predictive value 58.97%, negative predictive values 44.43%; GPC-3 not useful as diagnostic and prognostic marker for HCC
Xia et al. (2012)	HA	152 small HCC underwent complete radiofrequency ablation	High HA levels are main prognostic factors of local recurrence after complete radiofrequency ablation
Metwaly et al. (2012)	SDC-1	40 HCC vs. 31 liver cirrhosis vs. 15 controls	128.64 ± 16.45 ng/ml vs. 83.23 ± 10.13 ng/ml vs. 31.52 ± 15.3 ng/ml ($p < 0.05$)
Sadik et al. (2012)	HA	19 noncirrhotic HCC vs. 50 cirrhotic HCC vs. 36 cirrhosis vs. 21 controls	HA levels significantly increased in all three patients' groups
Abdelgawad et al. (2013)	GPC-3	40 HCC vs. 10 cirrhosis vs. 10 controls	Diagnostic values: Sensitivity 95%, Specificity 95%, Diagnostic accuracy 95%, Positive predictive value 97.5%, Negative predictive value 90.5%; promising diagnostic marker with high sensitivity and specificity for HCC
Nault et al. (2013)	Endocan, SDC-1, and GPC-3	58 early HCC vs. 67 advanced HCC vs. 170 alcoholic cirrhosis	Serum endocan and SDC-1 are prognostic serum biomarkers of overall survival in alcoholic cirrhosis with and without HCC
Li et al. (2013)	GPC-3	605 HCC vs. 25 controls	20.20 ± 5.41μg/l vs. 1.92 ± 0.95μg/l, $p < 0.01$
Yao et al. (2013)	GPC-3	123 Hepatocellular Carcinoma vs. 70 liver cirrhosis vs. 70 chronic hepatitis vs. 56 acute hepatitis vs. 50 nonliver tumor vs. 30 healthy controls	Diagnostic values: Sensitivity 52.8%, Specificity 98.8%, Diagnostic accuracy 83.5%, Positive predictive value 95.6%, Negative predictive value 80.7%
Chen et al. (2013)	GPC-3	155 HCC vs. 180 chronic hepatitis vs. 124 liver cirrhosis vs. 442 non-HCC cancer vs. 136 healthy controls	99.94 ± 267.2 ng/ml vs. 10.45 ± 46.02 ng/ml ($p < 0.0001$) vs. 19.44 ± 50.88 ng/ml ($p = 0.0013$) vs. 20.50 ± 98.33 ng/ml ($p < 0.0001$) vs. 4.14 ± 31.65 ng/ml ($p < 0.0001$); Application as a single marker in the diagnosis of HCC limited by sGPC-3 presence in lung cancer and thyroid cancer

(continued)

Table 6.4 (continued)

References	PGs/GAGs	Casuistry	Main findings
Lee et al. (2014)	GPC-3	120 HCC vs. 40 chronic liver disease	75.8 ng/ml (21.7–482.5 ng/ml) vs. 66.4 ng/ml (2.33–66.4 ng/ml) ($p < 0.020$)
Abd El Gawad et al. (2014)	GPC-3	40 HCC vs. 10 cirrhosis vs. 10 healthy controls	7.7 ng/ml (4.9–11 ng/ml) vs. 2.74 ng/ml (1.99–5.93 ng/ml) vs. 0.99 ng/ml (0.86–1.67 ng/ml) ($p < 0{,}0001$); Diagnostic values: Sensitivity 95%, Specificity 95%, Diagnostic accuracy 95%, Positive predictive value 97%, Negative predictive value 90.5%
Wang et al. (2014b)	GPC-3	84 HCC vs. 80 cirrhosis vs. 32 hepatitis B vs. 61 healthy controls	GPC-3 tests negative in all 84 HCC patients
Badr et al. (2014)	GPC-3	30 HCC vs. 30 hepatitis C virus (HCV) cirrhosis vs. 20 healthy controls	551.47 ± 185.25 ng/ml vs. 98.23 ± 73.54 ng/ml ($p < 0.01$); Diagnostic values: Sensitivity 100%, Specificity 93.3%, Diagnostic accuracy 96.7%, Positive predictive value 93.8%, Negative predictive value 100%
Mima et al. (2014)	HA	506 patients	High preoperative serum HA levels predict poor prognosis in patients with HCC after hepatic resection
Yu et al. (2015)	GPC-3	192 HCC vs. 54 gastric cancer vs. 92 colorectal cancer vs. 57 hepatitis vs. 31 esophageal cancer vs. 44 cirrhosis vs. 48 controls	GPC-3 levels in HCC significantly higher than that in other liver diseases as well as healthy controls
Haruyama et al. (2015)	GPC-3 N-terminal subunit antigen (sGPC-3N)	115 HCC vs. 25 controls	sGPC-3N levels significantly higher in HCC with respect to controls ($p < 0.0001$); high pre-operative sGPC-3N associated with shorter OS and DFS after hepatectomy ($p < 0.01$)
Jia et al. (2016)	GPC-3	283 HCC vs. 445 chronic hepatic diseases vs. 162 controls	Higher levels in both HCC and liver cirrhosis than controls ($p < 0.05$); low diagnostic potential alone but useful in combination with alpha-fetoprotein
Shimizu et al. (2016)	HA	400 HCC underwent liver resection	High HA levels (>200 ng/ml) as useful predictor of postoperative large amount of ascites, which are associated with poor prognosis

Jeon et al. (2016)	GPC-3	157 newly diagnosed HCC vs. 156 liver cirrhosis	0.80 ng/ml (0–3.09 ng/ml) vs. 0.60 ng/ml (0.07–7.40 ng/ml) ($p = 0.255$)
Attallah et al. (2016)	GPC-3	138 HCC vs. 56 liver cirrhosis vs. 62 liver fibrosis	Diagnostic power: sensitivity 93%, specificity 93%, positive predictive value 89%, negative predictive value 95%, efficiency 93%
Jing et al. (2017)	GPC-3	39 HCC vs. 31 liver cirrhosis	Higher GPC-3 levels in HCC ($p < 0.0001$); Diagnostic values: Sensitivity 89.74%, Specificity 96.77%, Positive predictive value 97.2%, Negative predictive value 88.2%; effective noninvasive diagnostic indicators of HCC
Sun et al. (2017)	GPC-3	76 HCC vs. 30 Cirrhosis vs. 40 Chronic liver disease vs. 30 controls	272.5 ± 13.3 ng/ml vs. 661.9 ± 6.8 ng/ml vs. 57.5 ± 8.3 ng/ml vs. 56.2 ± 6.1 ng/ml ($p < 0.01$)
Ofuji et al. (2017)	GPC-3	25 stage I HCC who underwent surgical resection	33.7 ng/ml (7.5–1729.3 ng/ml) (postoperation) vs. 60.1 ng/ml (6.4–3466.8 ng/ml) ($p < 0.001$); Postoperative recurrence associated with high preoperative plasma GPC-3
Farag et al. (2018)	GPC-3	145 HCC vs. 105 chronic liver cirrhosis vs. 50 healthy controls	Diagnostic values: Sensitivity 95%, Specificity 100%, Diagnostic accuracy 95%, Positive predictive value 97.5%, Negative predictive value 90.5%
Wu et al. (2019)	Fucosylated GPC-3 (fuc-GPC-3)	51 HCC vs. 47 benign liver disease	fuc-GPC-3 effective and useful tumor biomarker
Tahon et al. (2019)	GPC-3	40 cirrhotic patients with primary HCC vs. 30 cirrhotic patients without HCC vs. 15 healthy controls	12.08 ± 14.11 ng/ml vs. 2.07 ± 1.44 ng/ml vs. 1.25 ± 0.32 ng/ml ($p < 0.001$)
Hagag et al. (2020)	GPC-3	25 HCC vs. 75 liver cirrhosis vs. 50 controls	Higher serum levels of GPC-3 in HCC ($p < 0.001$); positive correlation between GPC-3 and miR-1291
Liu et al. (2020)	GPC-3	210 HCC vs. 36 intrahepatic cholangiocarcinoma vs. 9 combined Hepatocellular cholangiocarcinoma vs. 10 metastatic liver cancer vs. 134 normal controls	Serum GPC-3 superior to AFP for the diagnosis of early-stage HCC

(continued)

Table 6.4 (continued)

References	PGs/GAGs	Casuistry	Main findings
Tang et al. (2020)	GPC-3	166 HCC vs. 94 health controls vs. 50 benign controls	0.210 (0.048, 0.801) mg/l vs. 0.029(0.019, 0.052) mg/l vs. 0.033(0.021, 0.043) mg/l ($p < 0.001$)
Shimizu et al. (2020)	GPC-3	25 hepatitis C virus-related HCC vs. 15 hepatitis B virus-related HCC vs. 16 HCC	9.9 pg/ml (2.8–273 pg/ml) vs. 2.6 pg/ml (0.5–384 pg/ml) vs. 3.0 pg/ml (0.5–22.1 pg/ml); association between GPC-3 expression and secretion and the virus type

Table 6.5 Meta-analysis studies on the diagnostic significance of high serum GPC-3 levels in HCC patients

References	Casuistry	Assay	Sensitivity	Specificity	Positive likelihood ratio (PLR)	Negative likelihood ratio (NLR)	Diagnostic odds ratio (DOR)
Xu et al. (2013)	590 HCC patients/10 studies	ELISA/ radioimmunology	0.59 (95% CI: 0.55–0.63)	0.85 (95% CI: 0.82–0.87)	6.290 (95% CI: 2.649–14.933)	0.488 (95% CI: 0.386–0.617)	17.988 (95% CI: 5.361–60.353)
Liu et al. (2014)	898 HCC patients/12 studies	ELISA/ radioimmunology	0.53 (95% CI: 0.49–0.57; I^2: 79.9%)	0.77 (95% CI: 0.74–0.81; I^2: 95.9%)	NR (I^2: 91.5%)	NR (I^2: 86.1%)	10 (95% CI: 2–38; I^2: 90.6%)
Yang et al. (2014)	NR/18 studies	ELISA/ Chemilumi-nescent immunoassay	0.69 (95% CI: 0.55–0.80; I^2: 90.71%)	0.93 (95% CI: 0.85–0.97; I^2: 97.30%)	10.50	0.34	31 (95% CI: 11–92)
Jia et al. (2014)	1935 HCC patients/19 studies	ELISA/ radioimmunology	0.55 (95% CI: 0.53–0.57)	0.84 (CI: 0.82–0.86)	5.22 (CI: 3.09–8.84)	0.54 (CI: 0.46–0.63)	13.8 (95% CI: 6.6–28.8)
Liu et al. (2015)	1201 HCC patients/17 studies	ELISA/ Chemilumi-nescent immunoassay	0.56 (95% CI: 0.53–0.59; I^2: 90.7%)	0.89 (95% CI: 0.87–0.90; I^2:94.3%)	7.82 (CI: 3.86–15.85; I^2: 93.9%)	0.48 (CI: 0.39–0.59; I^2: 85.9%)	26.73 (95% CI: 10.31–69.26; I^2: 90.0%)
Xu et al. (2019)	11 studies	ELISA	0.55 (95% CI: 0.52–0.58; I^2: 93.3%)	0.58 (95% CI: 0.54–0.61; I^2: 90.2%)	1.69 (95% CI: 1.20–2.39)	0.67 (95% CI: 0.50–0.90)	3.64 (95% CI: 1.74–7.60; I^2: 85.3%)

Positive likelihood ratio (PLR): true positive/false positive
Negative likelihood ratio (NLR): false negative/true negative
Diagnostic odds ratio (DOR): the odds of positive test results in patients with HCC/ the odds of positive test results in patients without HCC
I^2: a quantitative measurement of inconsistency across different studies. I^2 value typically ranges from 0 (no observed heterogeneity) to 100% (maximal heterogeneity), and an I^2 value $\geq$50% is considered to represent substantial heterogeneity
NR Not reported

values, being sensitivity, specificity, positive predictive value, and negative predictive value 89.74%, 96.77%, 97.2%, and 88.2%, respectively. Similar results have been obtained by Farag et al. (2018) by analyzing GPC-3 levels in 145 HCC patients, 105 with chronic liver cirrhosis vs. 50 healthy controls (Farag et al. 2018).

Interestingly, Ofuji et al. (2017) reported an association between perioperative serum GPC-3 levels and tumor recurrence rate in 25 patients with stage I HCC who underwent surgical resection (Ofuji et al. 2017). However, Shimizu et al. (2020) who recently evidenced a positive correlation among serum GPC-3 levels, its expression in HCC, and the comorbidity with hepatitis C virus infection in 56 patients affected by HCC, suggested the utility of serum GPC-3 (as predictive of tissue GPC-3) to select the most appropriate therapy (e.g., immunotherapies targeting GPC-3) (Shimizu et al. 2020). Overall, all the mentioned studies have highlighted the usefulness of serum GPC-3, in combination with α-fetoprotein, as an effective noninvasive diagnostic and prognostic marker of HCC.

6.3.2 Multiple Myeloma

MM is a hematological cancer characterized by uncontrolled proliferation of malignant plasma cells that spread throughout the skeletal system where they crowd out normal blood cells disrupting hematopoiesis and bone physiology and creating lytic lesions. Although the etiology of MM is unknown in most patients, it may evolve from benign monoclonal gammopathy and some risk factors including obesity, alcohol consumption, radiation exposure, and family history have been reported (International Agency for Research on Cancer (IARC) 2020).

Despite the many progresses that have been done in treatment because of its capacity to develop drug resistance, MM is still incurable, as it ultimately leads almost all patients to death, with a median survival of 4–6 years. MM is characterized by a strong interaction between tumor cells and bone marrow microenvironment that supports their survival and growth through signals mediated by adhesion molecules, cytokines, and growth factors (Hideshima et al. 2007).

It is well known that SDC-1 is involved in multiple cell processes, including proliferation, migration, adhesion, and angiogenesis. In this respect, it may act both as coreceptor for various Hep-binding growth factors, such as bFGF/FGF2, VEGF, TGF-β, as well as PDGF, and as receptor primarily through its HS chains. Dysregulation of its expression, as well as its shedding, may lead to cancer progression in different ways, as recently reported by Teixeira and Götte (2020). Generally, if downregulated, it may enhance cell motility and invasion by reducing cell–matrix interactions, whereas in some cases, its upregulation may contribute to angiogenesis (Akl et al. 2015). Furthermore, the release of its ectodomain by the activity of sheddases, such as MMPs and heparanase, promotes stemness and tumorigenesis (Ramani et al. 2013; Tripathi et al. 2020; Teixeira and Götte 2020). Indeed, the free extracellular moiety of SDC-1 is still bioactive, modulating the binding between growth factors and their receptors in tumor stroma, which acts as a storage of growth

factors and chemokines promoting cell proliferation and tissue invasion, as well as sequestering inhibitory molecules (Akl et al. 2015).

Since SDC-1 plays key roles in maturation of normal B cells, as well as in their differentiation and function, its expression is tightly regulated (Reijmers et al. 2013). Besides, SDC-1 is essential for the survival of long-lived plasma cells and MM plasma cells (Mahtouk et al. 2006) which express it at high levels. The shedding of SDC-1, carried out by matrix proteases and/or endoglycosidases, releases in plasma its ectodomain and bioactive HS fragments of 5–10 disaccharides. In this respect, the high expression of heparanase, compared to normal tissues, is strongly correlated with a poor prognosis (Mahtouk et al. 2007; Purushothaman et al. 2010; Tripathi et al. 2020; Purushothaman and Sanderson 2020), representing a potential therapeutic target. Actually, new heparanase inhibitors, such as SST0001 developed to prevent glycocalyx degradation, are promising candidates for inhibiting myeloma growth (Ritchie et al. 2011).

Numerous studies have shown that serum SDC-1 levels, which are physiologically low in healthy people, following glycocalyx shedding are elevated in plasma of patients with MM (Table 6.6). Overall, these studies show that soluble SDC-1 represents a strong independent prognostic factor (Seidel et al. 2000; Bjoro et al. 2018) associated with both poor survival and resistance to chemotherapies (Aref et al. 2003; Janosi et al. 2004; Lovell et al. 2005). Nonetheless, its involvement in the crosstalk between MM cells and bone marrow microenvironment makes it also a promising molecular target for therapeutic strategies (Yang et al. 2007; Katz 2010; Ritchie et al. 2011; Reijmers et al. 2013).

6.4 Conclusions

It is currently and widely accepted that ECM, besides being a key modulator of cell–cell communication and cell function, greatly contributes to tissue homeostasis as well as to pathogenesis of several diseases, including cancer. HSPGs in particular, as major functional ECM components, may alter many critical cell processes including proliferation, transformation, and tissue invasion during tumorigenesis. Accordingly, they can be highly and specifically expressed in some tumors, representing useful diagnostic markers and potential therapeutic targets. During inflammation, the shedding by matrix enzymes releases in circulation bioactive fragments that, in turn, can be potentially valuable as noninvasive early markers. However, among the large number of published studies, data are sometimes conflicting or of low clinical relevance. In this respect, the absence of both common experimental design including standard protocols for enrollment and accurate diagnostics kits represent confounding issues that have to be solved.

Table 6.6 Main findings of studies on the association of serum PGs/GAGs levels with MM, carried out in the last 20 years

References	PGs/GAGs	Casuistry	Main findings
Dahl et al. (1999)	HA	386 MM (58% in stage III, 33% in stage II, and 9% in stage I)	Patients with abnormally low or high HA levels had more advanced disease and a shorter median survival
Seidel et al. (2000)	SDC-1	174 MM vs. 40 healthy controls	643 units/ml (401–2022 units/ml) vs. 128 units/ml (76–208 units/ml), $p < 0.0001$; survival: 20 months vs. 44 months ($p < 0.0001$)
Aref et al. (2003)	SDC-1	25 newly diagnosed MM	Soluble SDC-1 higher in nonresponders to chemotherapy vs. responders ($p < 0.01$), and in nonsurvivors vs. survivors ($p < 0.001$); Plasma cells SDC-1 expression lower in nonresponders to chemotherapy vs. responders ($p < 0.01$), in nonsurvivors vs. survivors ($p < 0.05$)
Kyrtsonis et al. (2004)	SDC-1	27 MM vs. 11 healthy controls	177.5 ng/ml (34–3500 ng/ml) vs. 40 ng/ml (28–75 ng/ml), $p = 0.001$; correlation with stage and shorter survival
Kumar et al. (2004)	SDC-1	501 MM	Survival: SDC-1 > 158 ng/ml vs. SDC-1 < 158 ng/ml, 36.3 months vs. 49.3 months ($p < 0.0001$)
Janosi et al. (2004)	SDC-1	13 monoclonal gammopathy of undetermined significance vs. 4 solitary plasmocytoma vs. 50 MM; 6 months follow-up of MM patients receiving chemotherapy	77.9 ng/ml (33–122 ng/ml) vs. 65.6 ng/ml (33.8–94.5 ng/ml) vs. 223.8 ng/ml (36–508 ng/ml); Responders to chemotherapy ($n = 11$): 106 ng/ml (57.3–440 ng/ml) (after 6 months) vs. 258 ng/ml (97.3–460 ng/ml) (baseline), nonresponders ($n = 4$): 361.7 ng/ml (251–486 ng/ml) (after 6 months) vs. 327 ng/ml (245–466 ng/ml) (baseline)
Andersen et al. (2005)	SDC-1	67 newly diagnosed MM vs. 18 controls	1053.92 ± 292.72 ng/ml vs. 81.28 ± 8.83 ng/ml, $p < 0.0001$; SDC-1 is a strong independent prognostic factor of shorter survival

(continued)

Table 6.6 (continued)

References	PGs/ GAGs	Casuistry	Main findings
Lovell et al. (2005)	SDC-1	324 MM at presentation vs. 154 MM at plateau phase	336 ng/ml (143–1635 ng/ml) vs. 192 ng/ml (111–422 ng/ml) ($p < 0.0006$)
Schaar et al. (2005)	SDC-1	66 MM vs. 54 monoclonal gammopathies of undetermined significance (MGUS) vs. 69 provisional MGUS (no bone marrow examination performed) vs. 36 controls	SDC-1 levels widely variable and of limited discriminatory value in patients with newly diagnosed monoclonal proteinemia
Janosi et al. (2005)	SDC-1	MM vs. plasmocytoma vs. MGUS	Strong independent indicator of poor prognosis
Maisnar et al. (2006)	SDC-1	17 MM vs. 14 MGUS	1542 ng/ml (10–17,300 ng/ml) vs. 32 ng/ml (5–128 ng/ml), $p < 0.001$; high levels associated with worse prognosis in MM
Kim et al. (2010)	SDC-1	28 MM vs. 50 controls; 6-month follow-up of MM patients receiving chemotherapy	265 ng/ml (98–1049 ng/ml) vs. 81 ng/ml (27–192 ng/ml) ($p < 0.0001$); responders to chemotherapy ($n = 20$): 97 ng/ml (60–470 ng/ml) (after 6 months) vs. 220 ng/ml (98–1,049 ng/ml) (baseline), nonresponders ($n = 8$): 266 ng/ml (172–629 ng/ml) (after 6 months) vs. 337 ng/ml (265–785 ng/ml) (baseline)
Scudla et al. (2010)	SDC-1	179 MM vs. 89 MGUS	189.1 (2.5–256.0) ng/ml vs. 42.2 (2.5–256.0) ng/ml ($p < 0.00001$)
Skliris et al. (2011)	Serglycin	37 MM vs. 21 healthy controls	Higher levels in MM patients ($p = 0.0001$)
Minarik et al. (2012)	SDC-1	156 MM vs. 88 MGUS	Serum SDC-1 correlates with the activity of MM and might become useful in differentiation of MGUS, asymptomatic MM, and overt/symptomatic form of MM
Gupta et al. (2015)	Versican (VCAN)	25 MM vs. 10 idiopathic thrombocytopenic perpurae vs. 25 healthy controls	Higher levels of VCAN in MM also in relation with disease severity; potential marker of active disease
Cigliana et al. (2015)	SDC-1	50 Intact Immunoglobulin MM vs. 34 Light Chain MM vs. 40 healthy controls	90.73 ± 103.80 ng/ml vs. 40.21 ± 55.22 ng/ml vs. 15 ± 9.02 ng/ml ($p = 0.012$; $p < 0.0001$; $p = 0.006$)

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Chapter 7
Hyaluronan in the Extracellular Matrix of Hematological and Solid Tumors. Its Biological Effects

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Abstract Tumors are most frequently induced by environmental factors with oncogenic potential. Beside tumor cells, cancer tissue contains several nonmalignant cells with well-defined function, embedded into the acellular extracellular matrix playing an important role in tumor progression. The extracellular matrix (ECM) is an indispensable element of all tissues which can modulate cell growth, survival, migration, immune response, and drug resistance. Among its main molecular components are glycosaminoglycans (GAGs) and proteoglycans (PGs), which have been shown to play fundamental roles either in physiological or malignant tissue. An important GAG of the ECM in the tumor microenvironment (TME) is hyaluronic acid, whose synthesis and degradation are altered in a variety of tumors. The biological effects of the abnormal accumulation of HA and the subsequent remodeling of the associated ECM differ according to the type of tumor. In this sense, the ECM-derived HA biological effects in different types of tumors are discussed below in this chapter. Additionally, we make it clear that approaches that interfere with HA metabolism may be a potential therapeutic approach for the treatment of cancer.

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7.1 Introduction

The tumor microenvironment (TME) is essential part of tumors, being either solid, as carcinomas, sarcomas, lymphomas, or liquid as leukemia. Within this, TME are composed by an extracellular matrix (ECM) which altered composition directly influences in malignant transformation and/or progression. The components from ECM as proteins, glycosaminoglycans (GAGs) and proteoglycans (PGs), have been shown to play fundamental roles in different physiological processes and malignancies. Among the GAGs, HA, which is our molecule of discussion during this chapter, is a key factor that organized the integrity in the ECM. HA is a member of GAGs family and is synthesized by all vertebrate organisms. It is present in almost all mature tissues, within the interstitial matrix but also at intracellular level. Besides, HA has multiple interactions with other proteins and PGs that also impact the function of ECM (Wei et al. 2020). Stavros Garantziotis and Rashmin C. Savani identified at least four distinctive characteristics of HA that defined its mechanism of action, its structure (weight and size), its binding patterns and chemical modifications, its metabolism, and the induction of intracellular signal (Garantziotis and Savani 2019).

The HA's chemical structure is well defined, it is a linear polysaccharide composed of 2000–25,000 repeating disaccharide units [D-glucuronic acid β (1→3) and *N*-acetyl-D-glucosamine β (1→4)] (Fig. 7.1). This large glycosaminoglycan (GAG) exhibits a molecular weight ranging from 10^5 to 107 Da (Laurent and Fraser 1992).

The large quantity of HA in the mammalian ECM impacts in hydration, lubrication, and physical properties of tissues (Toole 2004). In addition, it mediates cell proliferation and differentiation, regulates cell adhesion and motility, making it not only a structural component, but also an effective signaling biomolecule. In many types of tumors, with enhanced HA production, HA surface binding generate a physical barrier, which covers tumor cells and may contribute to the failure of treatments.

HA has a high rate of turnover in vertebrates, for example, in human, 5 g of the 15 g of total body HA renew daily (Stern 2008). It is synthesized by one of the three hyaluronan synthases (Has1/Has2/Has3). These are multipass transmembrane

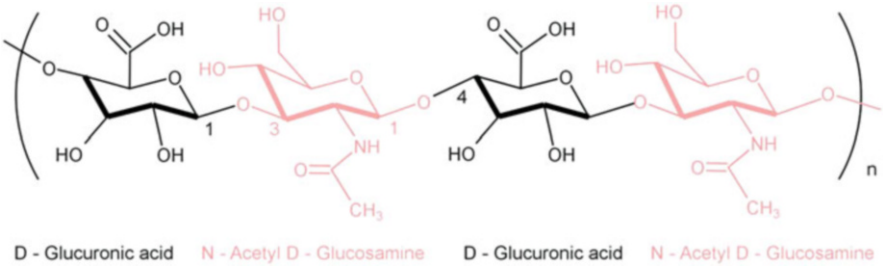

Fig. 7.1 Hyaluronic acid chemical structure. Disaccharide unit [D-glucuronic acid β (1→3) and *N*-acetyl-D-glucosamine β (1→4)] that forms the linear nonsulfated polysaccharide

enzymes that extrude HA while it is being synthesized either onto the cell surface or into the ECM (Monslow et al. 2015). Elevated levels of HA are associated with hyperproliferative and malignant phenotypes in melanomas and various carcinomas (Chanmee et al. 2016).

The breakdown of HA (HMW) in tissues can be accomplished by other mechanisms, either enzymatically by hyaluronidases, or by cleavage that are nonenzymatic oxidation reactions. In cancer cells, the type and levels of hyaluronidases are variable; in some cases, it is elevated, in others suppressed, compared to normal tissues (Toole 2004). A result of hyaluronidase activation, low molecular HA (LMW) is present in the ECM, which promotes inflammation, immune cell recruitment, and the epithelial cell migration. On contrary, elevated HMW-HA production, in the absence of fragmentation, is linked to cancer resistance (Toole 2009). Interestingly, Xiao Tian et al. have been observed that naked mole-rat fibroblasts secrete extremely high-molecular-mass HA, which is over five times larger than human or mouse HA. This sort of high-molecular-mass HA accumulates, abundantly in naked mole-rat tissues, providing cancer resistance and longevity to this species (Tian et al. 2013). Additionally, the authors observed that inhibition of HA synthesis enhances in these cells the susceptibility to malignant transformation.

HA interacts with cell surfaces in at least two ways. First, it can bind to specific cell surface receptors, to induce transduction of several intracellular pathway directly or by interacting with other receptors. HA interacts with many proteins, so termed as hyaladherins, several of which are known as surface receptors. The interaction depends on a binding domain presents every hyaladherins that contains seven nonacidic amino acids. This domain is common to all CD44 isoforms (CD44s and CD44v), the lymphatic vessel endothelial HA receptor-1 (LYVE-1), RHAMM (Receptor for hyaluronate-mediated motility), HARE (Hyaluronan receptor for endocytosis), and TOLL4, Stabilin (Orian-Rousseau 2010). Interestingly, layilin is a transmembrane protein widely expressed in different cell types and tissue extracts. It is found in peripheral ruffles of spreading. Layilin colocalizes with talin in ruffles and binds to talin's ~50-kDa head domain (amino acids 280–435), thus represents a membrane-binding site for talin in cell ruffles (Bono et al. 2001). However, this protein has not sequence homology to the other HA receptors and represents a novel member of the HA-binding protein family (Bono et al. 2001).

Among them, CD44 and RHAMM are the best characterized receptors to date as modulators in cancer. CD44 is a single-pass, glycosylated class-I transmembrane protein involved in multiple cellular functions, including interaction with the matrix microenvironment and intracellular signaling. The extracellular portion of CD44 primarily binds to glycosaminoglycan HA, thereby contributing to cell adhesion, migration, angiogenesis, inflammation, wound healing, and downstream signaling that promotes cell growth and survival (Ponta et al. 2003; Nikitovic et al. 2015; Karamanos et al. 2021).

Many studies have found a correlation between HA expression and the malignant properties of diverse tumors, and disruption of HA production or receptor interaction decreased tumor growth (Hosono et al. 2007; Stern 2008). In fact, the interaction of HA with its receptors drives to the activation of oncogenic pathways as the MAP

kinases and PI3K kinase/Akt inducing survival and cell proliferation, as well as various transport that participate in drug resistance and malignant cell properties (Toole and Slomiany 2008; Bouguignon 2009). Various adaptor proteins such as Vav2, Grb2, and Gab-1 mediate interaction of CD44 with upstream effectors as RhoA, Rac1, and Ras, which drive these pathways (Bourguignon 2008).

7.1.1 HA and Tumor Progression

HA participates in cancer initiation and tumor progression (Chanmee et al. 2016). The increased deposition of HA in tumor tissues is not an inactive process, rather, it triggers signaling events and promotes the association between CD44 and other cell surface receptors, driving to the activation of antiapoptotic pathways mediated by receptor tyrosine kinases that finally promote survival of tumor cells (Bourguignon 2008). Therefore, increase of HA relates to poor patient prognosis and facilitates tumor proliferation, invasion, and angiogenesis among others (Toole and Slomiany 2008). However, contradictory data also exist. In some contexts, accumulation of HA decreases tumorigenicity (Chanmee et al. 2016), while the expression of hyaluronidases, enzymes that degrade HA, can stimulate dissimilar tumor behavior (Stern and Jedrzejas 2006).

HA Oligosaccharides (oHA) were reported to have suppressive effects on various malignant tumors via disruption of the interactions between HA and receptors. oHA has been shown to suppress PI3K/Akt signaling pathway and module cell behavior as growth, cell survival, and expression of multidrug-resistance genes. In some lymphoma model as well as in breast carcinoma, oHA were able to induce apoptosis and reduce tumor growth in vivo (Russo et al. 2008). Besides, it was reported that oHA disaccharides suppressed progression of bone metastasis in breast cancer (Urakawa et al. 2012).

Contrary, HMW-HA or native HA contributes to flexibility of extracellular matrices (Solis et al. 2012) which is important for regulating cell trafficking. Moreover, HA contributes to tumor resistance in normal tissues by anti-inflammatory and antiproliferative effects (Cowman et al. 2015). HMW-HA present in either the peri-tumoral stroma or tumor parenchyma possess several functions that favor tumor growth by protection from apoptosis or inhibition of autophagy, not only by stopping the synthesis of autophagosomes but also inducing their degradation (Kuang et al. 2007). Moreover, LMW-HA can activate signaling cascades that promote proliferation, cell migration, neo-angiogenesis, immune cell influx, and mesenchymal cell trafficking (Cyphert et al. 2015). LMW-HA also attracts macrophages, which in the microenvironment, polarize into subpopulation M2 that protect tumor cells from adaptive immune cell killing (Kuang et al. 2007). However, the HA MW-dependent actions can be controversial and are associated with the tumor context.

In the next sections, we will discuss into the different studies that demonstrate the participation of HA in tumor progression, making a distinction between solid tumors

and those of hematological origin, considering as liquids leukemias and myelomas, which do not produce solid masses, and lymphoma.

7.2 HA in Hematological Malignancy

Hematologic malignancies comprise a complex and diverse group of pathologies, including leukemia, myelomas, lymphomas that affect blood-forming cells. In this section, the features of each pathology will be summarized first, and then, the impact of HA and its main receptors CD44 and RHAMM on the progression of each kind of hematological cancer will be described.

7.2.1 HA and Leukemia

Leukemia is an uncommon and heterogeneous group of diseases characterized by infiltration of the bone marrow, blood, and visceral organs by neoplastic cells of the hematopoietic system (Menghrajani and Tallman 2018). Leukemia is among the ten types of cancer with high number of death in both sexes and all ages, according to The Globocan Cancer Observatory (https://gco.iarc.fr/today/data/factsheets/cancers/36-Leukaemia-fact-sheet.pdf). In general, leukemia is classified into lymphoid or myeloid (depending on the cell line of origin) and acute or chronic (according to the type of evolution), thus forming four main groups: Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), and Chronic Myeloid Leukemia (CML).

The ECM of the bone marrow (BM) is the largest anatomical component which contains factors that contribute to adhesion, functionality and even regulation of the hematopoietic stem cells (HSC) (Karantanou et al. 2018). In this way, HA, as part of this ECM, actively contributes to these effects. A decrease in the levels of HA in the BM impairs the ability of microenvironment to maintain normal hematopoiesis, and recruit mesenchymal hematopoietic cells into the medulla in alive (Goncharova et al. 2012; Khaldoyanidi et al. 2014). Similarly, the HA would appear to be critical in the niches of these cells as it would protect them from DNA damage by activating efflux pumps capable of extruding genotoxic compounds (Csoka and Stern 2013). In addition, the viscoelastic properties of HA contribute to the porosity and malleability of the ECM of stem cell niches which is important to resist somatic mutations and mechanical damage (Simpson et al. 2016; Gesteira et al. 2017). However, these properties are also used by tumor cells to establish multidrug resistance (García et al. 2009; Lompardía et al. 2013).

ALL results from the acquisition of mutations in hematopoietic progenitors that confer a proliferative and/or survival advantage and impair hematopoietic differentiation (Ley et al. 2013). Genetics is considered one of the main players in the etiology of acute leukemia (Tebbi 2021). However, other factors, such as age,

environment and occupation, infections, radiation, among others, have been implicated in leukemogenesis (Tebbi 2021). Interestingly, recent studies suggested that leukemia results from a type of gene–environmental interaction that can cooperate with a genetic predisposition, not by inducing mutations, but by reprogramming the epigenome to modulate gene expression (González-Herrero et al. 2018). ALL is diagnosed by the detection of equal or higher than 20% of blasts in the peripheral blood or bone marrow. However, this determination may not accurately define the biology of the disease (Menghrajani and Tallman 2018).

ALL is a hematological malignancy originating from B- or T-lymphoid progenitor cells and represents the most common type of cancer in children and adolescents (80% of cases). The 5-year survival rates of these groups of diseases have increased from 10 to 85% in the last 50 years. However, there are a significant number of relapses and patients who do not respond to therapy. In this sense, 20% of pediatric patients die from the disease and this number rises to 50% in adults (Terwilliger and Abdul-Hay 2017). The B-cell subtype (B-ALL) accounts for about 75–80% of ALL cases and it mainly develops in children with a peak incidence of around 2–5 years (Simioni et al. 2021). T-ALL represents 15–25% of all cases of acute leukemia of children and adults, respectively. Despite being the least frequent subtype, it results to be more aggressive than B-ALL and is considered a risk factor for poor prognosis for patients (Vadillo et al. 2018; Follini et al. 2019).

AML constitutes 15–20% of leukemia in children and adolescents and 80% of acute leukemia in adults (Fiegl 2016). There is a complex classification of AML that created by the combination of clinical, morphologic, immunophenotypic, and genetic features (according with WHO).

Both in bone marrow samples and in the serum of patients diagnosed with acute leukemia, HA levels are increased compared to samples of healthy patients. For this reason, HA has been proposed as a prognostic and follow-up marker for early detection of relapse (Sundström et al. 2005, 2010; Anagnostopoulou et al. 2017). CD44 and RHAMM are the HA receptors most studied in the context of ALL. CD44-HA interactions play a key role in the adhesion, homing, and migration of leukemia initiating cells in bone marrow niches (Zöller 2015; Schepers et al. 2015). Moreover, the ability of CD44 to interact with different components of the ECM is used by leukemic cells to maintain their niches favoring disease relapse and chemoresistance (Hanke et al. 2014; Zöller 2015; Izzi et al. 2017). Indeed, clinical experience indicates that high levels CD44 are associated with a poor prognosis and high relapse rates in AML patients (Hanke et al. 2014). In line with this, it was suggested that CD44 binds to HA promoting an inside-out activation of VLA-4 in AML cells, causing clustering and stabilization of the integrin, leading to the interaction with VCAM-1 in stromal cells. This AML cell–stromal cell interaction triggers survival signaling involving Akt, MAPK, and NF-kB pathway activation (Gutjahr et al. 2020). NOTCH1 plays a crucial role in T-ALL pathogenesis, as illustrated by the fact that over 60% of human T-ALLs contain gain-of-function NOTCH1 mutations that lead to ligand-independent NOTCH1 signaling (García-Peydró et al. 2018). In human T-ALL xenografts, it was demonstrated that CD44 is a direct NOTCH1 transcriptional target that mediates crucial cell interactions with the

BM microenvironment that result in preleukemic engraftment and further support T-ALL LIC (Leukemic Incited Cell) activity and disease progression (García-Peydró et al. 2018). For this reason, targeted therapy against CD44 aiming to block its interaction with ECM or decrease its expression in tumor cells has been studied (Gul-Uludağ et al. 2014; Vey et al. 2016; Amanzadeh et al. 2017). However, it is well known that different sizes of HA display different biological effects, it has been observed in B-precursor leukemia cells with high surface CD44 expression, that high levels of ultra LMW-HA (<10 kDa) trigger necrosis (Kasai et al. 2017).

Besides, RHAMM is considered a leukemia-associated antigen (Greiner et al. 2002) and it was proposed as an important molecular target for design of therapeutic vaccines in patients with AML (Schmitt et al. 2008; Willemen et al. 2016). A recent study showed that acute leukemia patients with high percentages of RHAMM-positive blasts had more postinduction blasts, blasts in minimal residual disease, and poorer prognosis (Shalini et al. 2018). However, little is known about the implication of this receptor in the biological mechanisms that contribute to the progression of the disease. Similarly, CD38 has become an interesting target for the treatment of ALL and HA its ligand (Lato et al. 2021). Daratumumab, a human monoclonal antibody that binds specifically to CD38, in addition to standard chemotherapy, is under investigation in a phase II trial for pediatric and young adult participants with relapsed and/or refractory T- or B-cell ALL (Clinical Trials. gov identifier: NCT03384654) (Lato et al. 2021). However, the implication of its interaction with HA in the progression of acute leukemia is an empty field for future research.

CLL is the most common leukemia in older patients. TP53 aberrations were associated with aggressiveness and resistance to therapy. For that reason, different first-line therapeutic strategies are used depending on the presence of mutation in TP53, as well as physical fit of patients (Hallek et al. 2018). However, the microenvironment also enhances CCL progression.

Certainly, *ex vivo* assays have established that CLL cells require immune or stromal cells in order to not die by apoptosis, showing the importance of CCL cells and their milieu interaction for leukemic cell survival (Collins et al. 1989). Moreover, HA and its receptors, CD44 and RHAMM, participate in CLL progression. Both CD44 and RHAMM were found to be increased in patients with CLL and have been associated with a worse prognosis, which is why they have been proposed as therapeutic targets (Giannopoulos et al. 2009; Tabarkiewicz and Giannopoulos 2010; Gutjahr et al. 2015). It was described that HA-RHAMM interaction, in presence of IL-8, is able to induce CCL cells migration (Till et al. 1999). Likewise, activation of CD40 enhances CD44 adhesion to HA promoting leukemic cells retention in lymph nodes and enhancing their proliferation and survival (Girbl et al. 2013). Moreover, CD44 is able to complex with key prognostic factors of CLL (CD38 and CD49d) providing a possible nexus between prognosis and leukemic biology (Gutjahr et al. 2015).

On the other hand, CML is a myeloproliferative neoplasm. It is characterized by the presence of Philadelphia chromosome, a product of the reciprocal translocation between chromosomes 9 and 22 (Apperley 2015). This aberration gives rise to a

fusion gene called BCR-ABL which encodes a constitutively activated kinase capable of activating multiple signaling pathways initiating the leukemogenic process due to the clonal expansion of malignant cells. Although tyrosine kinase inhibitor (TKIs) are very effective drugs and significantly increase the mean survival of patients, in many cases, the prolonged use of them leads to the selection of resistant leukemic cells by BCR-ABL-dependent mechanisms (gene amplification, protein overexpression, mutations in the active site of the enzyme) as well as by independent mechanisms (such as Pgp, PI3K, and ERK activation) (Bavaro et al. 2019). However, not only these stimuli favor drug resistance, but also the interaction with the bone marrow milieu enhances CML progression. In this sense, Graham et al. 2002 described that the leukemic stem cells of CML patients were resistant to imatinib and this resistance would be based on the niche in which they are found, which prevents the entry of drug, as well as provides survival signaling (Graham et al. 2002; Schepers et al. 2013).

Regarding HA, it also plays an important role in the progression of CML. In patients with therapeutic failure, after prolonged treatment with imatinib, a gelatinous transformation of the bone marrow characterized by high levels of HA has been described (Hong et al. 2010). In agreement, in vitro studies demonstrated that CML cell lines synthesize HA which interacts with CD44 and RHAMM, triggering PI3K/Akt and MEK/ERK signaling pathways avoiding the induction of senescence and favoring resistance to vincristine and imatinib (mediated by PI3K and Pgp activation) (Lompardía et al. 2013, 2019). It is worth to note that such proteins are involved in BCR-ABL-independent resistance to imatinib. Furthermore, the use of HA oligosaccharides (which are capable of binding to receptors without crosslinking them) allowed the sensitization of CML cell lines to imatinib by inducing apoptosis, as well as senescence through PI3K inhibition (Lompardía et al. 2016). In the same way, the inhibitor of HA synthesis, 4-methylumbelliferone, synergizes with imatinib inhibiting CML cell lines proliferation and enhances the induction of senescence (Lompardía et al. 2017, 2019). Considering the gelatinous transformation of bone marrow observed in patients with therapeutic failure and the in vitro findings described, it could be suggested that HA participates in resistance to the first-line drug. This appreciation is in concordance with in vitro studies which show that HA treatment was able to counteract the antiproliferative and pro-senescent effect of imatinib (Lompardía et al. 2019). In the same way, CD44 and RHAMM expression have been associated with worse prognostic and TKI resistance in CML patients (Greiner et al. 2002; Hu and Li 2016; Zhou et al. 2017). Therefore, reducing HA levels or mitigating its effects blocking CD44 and RHAMM would be a promising therapeutic approach to improve current therapy.

Considering that the bone marrow niche provides a sanctuary for leukemic stem cells since it favors immune and chemotherapeutic resistance, the looking for improvement in the leukemic therapy could result from understanding this complex microenvironment in which HA is a key player. In this way, the high levels of HA in bone marrow and the background described, let us to hypothesize that the leukemic cells are in a microenvironment that feedback their survival, favoring disease progression.

7.2.2 HA and Multiple Myeloma

Multiple myeloma (MM), a plasma cell neoplasia characterized by clonal proliferation of malignant plasma cells mainly in the bone marrow (BM). MM is an aggressive malignancy characterized by the clonal expansion of terminally differentiated B cells in the BM. It is the second most common hematological cancer. MM is clinically defined by increased BM plasmacytosis, serum and/or urine monoclonal immunoglobulin, secretion of free light chains, hypercalcemia, renal insufficiency, anemia, and bone pain due to osteolytic disease (Palumbo and Anderson 2011; Rajkumar et al. 2014).

Plasma cell's (PC) survival depends on different signals from neighboring cells within the BM (Slifka and Ahmed 1998). It was suggested that both the myeloma cells and the microenvironment have undergone alterations as early as during precursor stages of the disease (García-Ortiz et al. 2021). In addition, MM is associated with immune deficiencies, suggesting that the evolution of the disease from a precursor state is related with an immunosuppressive environment that allows tumor growth.

The interaction among myeloma cells and the components of the microenvironment is considered crucial in multiple myeloma pathogenesis. Myeloma cells make use of the supportive surrounding stromal cells, osteocytes, and endothelial cells for their own growth. Myeloma precursor cells have been shown to mediate progressive growth using in vivo experimental models that establish the relationship between the microenvironment and tumor signals in regulating tumor growth (Das et al. 2016). Adhesion molecules, cytokines, and the ECM play a critical role in the interplay among genetically transformed clonal plasma cells and stromal cells, leading to the proliferation, progression, and survival of myeloma cells.

The bone marrow microenvironment offers a structure, the ECM, which acts as a feeder and support for myeloma progenitor cells. ECM is constituted by fibronectin, collagen, osteopontin, HA, and laminin. Myeloma cells binding into ECM components proved to be important for survival and drug resistance (Katz 2010).

The glycosaminoglycan HA is a critical component of the hematopoietic microenvironment as mentioned above. It is possible that HA binds either growth factors or other ECM components. HA-expressing stromal cells create hematopoietic foci and act as feeder of hematopoietic cells in vitro (Goncharova et al. 2012). Moreover, B cells from MM patients showed HA matrix around the cells.

CD44/HA interactions are implicated in the regulation of homing of normal HSC and malignant cells into BM (Avigdor 2004; Krause et al. 2006). While myeloma cells migrate to the BM (Cook et al. 1997), their interactions with the microenvironment may significantly influence disease progression. CD44 and RHAMM are the major HA receptors of multiple myeloma cells. CD38, another hyaluronic acid interacting protein, is also expressed in high levels in myeloma cells (Costa et al. 2019). On the contrary, its expression is low in other lymphoid and myeloid cells making it an effective target for immunotherapies.

Vincent and Mechti demonstrate that IL-6, a growth factor for myeloma cells, greatly increases CD44 gene expression. The authors shown that IL-6 modulates CD44 RNA alternative splicing and induces the overexpression of all CD44 variant exons. As IL-6 secretion, induced from bone marrow stromal cells by myeloma cells, is mediated through direct cell-to-cell communication involving CD44 adhesion molecules. As consequence, it was suggested that a CD44/IL-6 amplification loop plays a crucial role in myeloma cell survival (Vincent and Mechti 2004).

In addition, adhesion of myeloma cells to the ECM increases angiogenesis, and the expression of adhesion molecules such as VLA4, LFA1, and CD44 are implicated in this event (Vacca et al. 1995). In some cases, the myeloma cells are able to degrade basement membrane and ECM. They must also reduce their affinity to ECM and cells, which are specific to the bone marrow, soon after upregulate migratory proteins. Subsequently, they alter their adhesion properties to extravasate and migrate through the ECM. Besides, in myeloma, plasma cells decrease expression of CD56 while increasing expression of certain CD44 isoforms that are important for proliferation and motility (Dahl et al. 2002). Finally, myeloma cells must degrade the basement membrane to allow passage through gaps of endothelial cells, and when arriving into the circulation, they may penetrate through the vasculature and form tumors in organs. Extramedullary growth is a feature of advanced MM but circulating tumor cells can also be detected even in early stages of MM.

The role for CD44 as a promoter of cell adhesion-mediated drug resistance has been described in several cancers, and it may in part mediate dexamethasone resistance in myeloma (Ohwada et al. 2008).

Lenalidomide therapy is used in frontline, relapsed/refractory, and maintenance settings for multiple myeloma. Lenalidomide-resistant models were found to overexpress the HA. In addition, resistant cells were more adhesive to bone marrow stroma and HA-coated plates. Blockade of CD44 with monoclonal antibodies, free HA, or CD44 knockdown reduce cellular adhesion and sensitized to lenalidomide by affecting Wnt/β-catenin signal. Bjorklund et al. showed a strong association between CD44 expression and clinical resistance to lenalidomide, suggesting that CD44 levels could serve as biomarkers of lenalidomide resistance (Bjorklund et al. 2014).

Inhibitions of the interactions of MM cells with the BM represent an interesting therapeutic strategy in MM (Bjorklund et al. 2019). The development of new tumor therapies uses HYAL as an adjuvant, agent to depolymerize HA from the ECM. As consequence of the degradation of HA, the absorption of the chemotherapeutic agent of choice is favored. To that end, the administration of Darzalex uses daratumumab plus human recombinant HYALs to treat multiple myeloma (Kaul et al. 2021).

7.2.3 HA and Lymphoma

Lymphoma is a group of hematological malignancies that begins in cells of the lymphoid system. The two main types are Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). The majority of lymphoma cases derive from the

B cell lineage and 90% of them originate from different stages of maturation and differentiation during their transit through the germinal center (GC) (Hamel et al. 2012). HL and different subtypes of NHL, such as Diffuse Large B Cell (DLBCL), Burkitt's Lymphoma (BL), and Follicular Lymphoma (FL), derive from this compartment (Klein and Dalla-Favera 2008).

It has been observed that an increase in the HA content in patient biopsies would correlate with high-grade lymphomas (Bertrand et al. 2005), indicating that different types of lymphoma cells produce dissimilar levels of HA and this fact would be related to their malignancy. Additionally, it has been demonstrated that lymphoma cells synthesize this GAG (Funamoto et al. 2002; Kuwabara et al. 2003). Among HA receptors, CD44 has been proposed as a diagnostic marker, as well as a therapeutic target in certain types of lymphomas (Ma et al. 2008; Eberth et al. 2010). In murine NHL lymphoma cell lines, the interaction of HA with CD44 would activate the NF-kB pathway and this would correlate with an increase in MMP-9 activity, which would favor cell invasion (Alaniz et al. 2004) Moreover, the inhibition of HA synthesis by 4-methylumbelliferone (4-MU) suppresses MMP-9 activity on lymphoma cells (Nakamura et al. 2007). On the other hand, blocking CD44-HA interaction with HA oligosaccharides inhibits the activation of the PI3K/Akt pathway and modulates efflux pumps sensitizing lymphoma resistant cells to chemotherapy treatment (Russo et al. 2008). In contrast, exogenous LMW-HA might increase intracellular doxorubicin accumulation in CD44 positive lymphoma cells by the modulation of ABC drug transporters expression involved in drug efflux in hematopoietic malignancies (Vitale et al. 2018). These data suggest that HA–CD44 interactions also play a key role in invasion and chemoresistance events in lymphoma and that different sizes of HA may exert dissimilar biological effects on lymphoma progression.

In cHL, HRS cells actively modulate their microenvironment to support their survival and proliferation, and to create an immunosuppressive environment (Weniger and Küppers 2021). In this context, it was suggested that HRS cells activates endothelial cells to increase HA levels in the perivascular zone which facilitate the recruitment of naïve T cells into lymph nodes through CD44-HA adhesive pathways (Fhu et al. 2014). Moreover, it was demonstrated that a downregulation of CD44 after inhibition of NF-kB signaling may decrease cHL cells adhesion to HL-associated fibroblast, highlighting the role of this receptor in with chemotherapy resistance (Celegato et al. 2014).

The literature regarding the role of HA in lymphoma progression is scarce. However, the interplay between HA and CD44 represent an interesting starting point to delve further in the studies of the biology of this GAG in the context of lymphoma and to investigate its potential as a new therapeutic target for this disease.

7.3 Biological Effects of HA from ECM in Solid Tumors: Carcinomas, Sarcomas, and Gliomas

Malignant transformation of epithelial cells results in the development of carcinomas, being the most frequent solid tumors. Sarcomas arise from bone and connective tissues, being the osteosarcoma the most common malignancy of bone cells. Gliomas, the primary brain tumors, are caused by decontrolled growth of glial cells, which are the supporting cells of neurons. In this section, we describe and discus the role of HA in the most frequent solids tumor mentioned.

7.3.1 Carcinomas

7.3.1.1 HA and Breast Cancer

After lung cancer, breast carcinomas are the second most leading cause of mortality in females and correspond to the most frequently diagnosed malignancy, affecting 2.3 million new cases each year. Hyaluronic acid (HA) is known to be involved in breast cancer progression to metastasis (Toole 2004; Eberth et al. 2010). In breast cancer, a dramatic HA increase is observed, and HA levels have been suggested to predict clinical outcome. Even more, it has been observed that high levels of HA, and in particular small HA oligosaccharides, are associated with poor prognosis (Auvinen et al. 2000) and have been detected that invasive breast cancer cells synthesize and accumulate larger amounts of HA compared to normal breast epithelial tissues (Li et al. 2007). In line with these antecedents, Corte et al. have compared the HA accumulation in early and later stage breast tumors, specifically in ductal carcinoma in situ (DCIS), DCIS with microinvasion and invasive carcinoma, for determining if altered HA production is linked to invasion events in breast cancer. They demonstrated that HA levels are significantly increased in late-stage DCIS compared to early-stage, suggesting a key role for this glycosaminoglycan in the early invasive stage of breast carcinomas (Corte et al. 2010). These antecedents added to the key role of HA in modulating the inflammation, angiogenesis and fibrosis in breast cancer (Heldin et al. 2013), processes involved in its progression to metastasis (Toole 2004). Thus the regulation of HA metabolism will be a potential therapeutic target for the treatment of breast cancer. Numerous studies have demonstrated that inhibition of HA synthesis reduces breast cancer tumor cell proliferation and migration (Kultti et al. 2009; Urakawa et al. 2012; Brett et al. 2018). Brett et al. showed that the invasive and metastatic potential of breast carcinoma cells correlates to the presence of a HA-rich pericellular matrix, and that inhibition of HA synthesis significantly inhibits carcinoma cell extravasation and invasion (Brett et al. 2018). Kultti et al. showed that the growth of MCF-7 cells is sensitive to inhibition of HA synthesis, and that inhibiting HA production inhibits migration of the noninvasive MCF-7 breast cancer cells (Kultti et al. 2009). Even more, Urakawa showed that

suppression of HA synthesis and accumulation by 4-MU inhibited cell proliferation, motility and invasiveness, and induced apoptosis in the highly aggressive MDA-MB-231 breast cancer cell line (Urakawa et al. 2012). Furthermore, inhibition of HA production or its degradation was shown to improve drug delivery in breast cancer (Shpilberg and Jackisch 2013; Kohli et al. 2014; Clift et al. 2019). In this sense, has been shown that the use of hyaluronidases to degrade HA in the TME improves drug delivery of antibody-based trastuzumab-targeted therapy in HER2+ breast cancer (Shpilberg and Jackisch 2013). Similarly, Kohli et al. showed a more heterogeneous distribution of Doxil and reduced tumor growth by inhibiting of HA synthesis (Kohli et al. 2014). Even more, a recent study has shown that HA degradation by pegrhyaluronidase allows to remodel the TME in a murine model of breast cancer increasing the uptake of anti-Programmed Death-Ligand 1 (PD-L1) therapeutic antibody and reducing the tumor growth (Clift et al. 2019). It has also been shown that HA allows to overcome the pro-apoptotic effects of SP-D surfactant protein D and can be used as an escape mechanism in breast cancer. In this sense, when a possible interaction between SP-D, with immune surveillance function against tumor cells, and hyaluronic acid, was studied, it was shown that, in the presence of HA, rfhSP-D is incapable of inducing apoptosis in triple positive breast cancer cell lines that overexpress HER2. In addition, HA-bound rfhSP-D was able to restore cell growth, suggesting that these breast cancer cells may use HA as an escape mechanism to overcome pro-apoptotic effects of surfactant protein D rfhSP-D (Murugaiah et al. 2020). Even more, Witschen et al. found that production of the chemokine CCL2 by breast cancer cells was significantly decreased after depletion of either CD44 or HA. In vivo, they found that CD44 deletion in breast cancer cells resulted in a delay in tumor formation and localized progression. This finding was accompanied by a decrease in infiltrating CD206+ macrophages, which are typically associated with tumor promoting functions (Witschen et al. 2020). Furthermore, a recent study has shown the importance of altering HA metabolism to malignant progression of breast carcinoma. Arnold et al. proved that increased HA synthesis requires reprogramming of the glucose metabolism and that disrupting of this metabolic reprogramming through the genetic and pharmacological depletion of the HA precursor UDP-glucuronic acid significantly reduces cellular invasion and colony formation in vitro in MDA-231 cells. Even more, results of in vivo mesenchymal-like breast cancer models revealed a reduction in tumor growth and metastasis (Arnold et al. 2020).

These data suggest that HA plays a key role in proliferation, invasion, and chemoresistance events in breast cancer and that it does so through different mechanisms.

7.3.1.2 HA and Ovarian Cancer

Epithelial ovarian cancer (EOC) is the commonest cause of gynecological cancer-associated death and the fourth cancer-related deaths in women in the developed world (Jayson et al. 2014). Approximately 90% of all ovarian cancers are epithelial

and of these ~80% represent the serous histological subtype (Hiltunen et al. 2002). Conventional ovarian cancer treatment comprises first-line chemotherapy with a carboplatin-paclitaxel regimen and cytoreductive surgery (Marchetti et al. 2010; Vaughan et al. 2011). However, although this regimen is initially effective, there is a high recurrence rate and most ovarian cancer patients suffer at least one relapse within 12–18 months (Colombo et al. 2006, 2017; Kartal-Yandim et al. 2016; Yousefi et al. 2016) owing to chemoresistance and metastasis (Lengyel 2010; Amoroso et al. 2017). Increased expression of HA has been shown to be closely correlated with the epithelial ovarian cancer (EOC) degree and metastatic potential (Anttila et al. 2000; Ween et al. 2011), with a 100-fold increase in HA expression in grade three EOC (Hiltunen et al. 2002). Different antecedents suggest that targeting the synthesis or accumulation of HA represents a promising therapeutic approach for the treatment of ovarian cancer. Lokman et al. showed that inhibition of HA production by 4-MU in combination with chemotherapy with carboplatin (CBP) significantly reduced chemo-resistant serous ovarian cancer cells survival and spheroid formation and increased apoptosis of this cells compared to CBP alone. Even more, showed that 4-MU alone or combined with CBP significantly decreased the invasion ability of these cells in vivo compared to control treatment. The fact that the inhibitor of the synthesis of HA 4-MU allows to overcome the chemoresistance to the chemotherapeutic drug CBP and that they determined that the production of HA increases in ovarian cancers resistant to chemotherapy indicates that the inhibition of HA is, therefore, a promising new strategy to overcome chemoresistance and improve ovarian cancer survival (Lokman et al. 2019). Even more, the reduction of HA-pericellular coat was related with the inhibition of cell migration, proliferation, and invasion (Anttila et al. 2000). On the other hand, clear-cell ovarian cancers, an epithelial ovarian cancer subtype, show a spherule-like mucoid stroma with a hollow acellular space. Despite the absence of stromal cells, both the mucoid stroma and hollow spheroids contain abundant ECM, mainly composed of HA that plays a crucial role in the formation of that structures and tumor progression. In this sense, Kato et al. showed that when HA synthesis was inhibited by 4-MU in HAC-2, a clear cell carcinoma cell line, the spherule-like accumulation of HA, or hollow spheroids were not observed. Moreover, the inhibition of HA synthesis was associated with the reduction of cell growth (Kato et al. 2016). Even more, Lin et al. found that upregulation of UDP-glucose dehydrogenase (UGDH) is related to ovarian cancer metastasis and that knockdown of UGDH significantly decreased wound healing and migration and proliferation in three ovarian cancer cell models, TOV21G, A2780, and HeyA8 cells. Furthermore, in an in vivo xenograft model from TOV21GHI cells, they demonstrated that UGDH removal significantly decreased ovarian cancer tumor growth (Lin et al. 2020). Given that the UDP-glucose dehydrogenase converts UDP-glucose to UDP-glucuronic acid, a precursor of several GAGs and PGs presents in the ECM, as well as cell proliferation and migration, it is likely that the elimination of UGDH decreased HA levels in ovarian cancer and thus affected the metastatic ability of ovarian cancer. However, further studies are needed to demonstrate the biological effects of HA from ECM in ovarian cancer.

7.3.1.3 HA and Prostate Cancer

Patients with clinically localized prostate cancer (CaP) are often treated with radical prostatectomy or radiation with curative intent. However, the disease recurs in a substantial number of the patients and becomes hormone refractory.

Lipponen et al. found high levels of HA in the stroma of prostate cancer and showed that stromal HA accumulation is related to poor prognosis in prostate cancer (Lipponen et al. 2001). Posey et al. showed that in radical prostatectomy specimens, HA expression is elevated in CaP tissues, but it is not an independent predictor of biochemical recurrence (Posey et al. 2003), besides has been observed that HA and HYAL-1 expression in prostate biopsy specimens and correlated in with disease recurrence (Gomez et al. 2009). Lokeshwar et al. studied the effects of inhibition of HA synthesis by 4-MU on five prostate cancer cell lines, LNCaP, DU145, PC3-ML, LAPC-4, and C4-2B. They demonstrated that 4-MU inhibited the proliferation of all cell lines by inducing apoptosis, in a dose-dependent manner, and that this effect of 4-MU on cell growth and apoptosis was due to the inhibition of HA synthesis. Even more, they demonstrated that 4-MU inhibited invasive activity and chemotactic motility of PC3-ML and DU145 cells by inhibiting HA production. In PC3-ML xenograft mice model, oral administration of 4-MU significantly inhibited tumor growth and reduced microvessel density in tumors from 4-MU-treated animals (Lokeshwar et al. 2010). In a transgenic adenocarcinoma of the prostate model, oral administration of 4-MU also significantly decreased microvessel density and prostate cancer cells proliferative index. Furthermore, daily gavage of 4-MU inhibited tumor growth in a DU145 subcutaneous xenograft model and inhibited skeletal metastasis in the jaw, pelvis, femur, and spinal cord in a PC3-ML intracardiac bone metastasis model. Even more, they showed that all these effects were the result of inhibition HA synthesis and signaling (Yates et al. 2015). Different studies showed that, in addition to the accumulation of HA in the tumor stroma, the alteration of hyaluronic acid synthase and hyaluronidase in tumor epithelial cells are associated with increased cell proliferation, invasion, metastasis, and poor outcome in men who have undergone radical prostatectomy (Simpson and Lokeshwar 2008; Bharadwaj et al. 2009).

7.3.1.4 HA and Pancreatic Cancer

Pancreatic cancer is one of the most difficult conditions to treat, although it only accounts for 4% of all cancers; 5-year survival is less than 10% in patients with the disease and has remained relatively unchanged over the past 25 years (Siegel et al. 2016). Most patients present with locally advanced or metastatic disease, and such individuals have a grim median survival of 6–10 months, and 3–6 months, respectively. Although 10–15% of patients have potentially rejectable tumors, the recurrence rate of the disease following surgery is high (Wong and Lemoine 2009). The

vast majority (90%) of pancreatic cancers (PC) are tumors originating from pancreatic ductal cells (Sener et al. 1999; Sharma et al. 2011).

The most common subtype of human pancreatic malignancies is Pancreatic Adenocarcinoma (PDAC). This type of cancer originates in the ductal epithelium and evolves from premalignant lesions to fully invasive cancer. It has been established that PDAC is preceded by the evolution of precursor lesions called pancreatic intraepithelial neoplasia (PanIN 1A/B, 2, and 3) (Hruban et al. 2001), and, under certain conditions, Acinar Ductal Metaplasia (ADM) might be critical for the development of PanIN lesions (Means et al. 2005). The progression from histologically normal ductal epithelium to low-grade PanIN to high-grade PanIN is associated with the accumulation of specific genetic changes (Guerra et al. 2007; Hidalgo 2010). It has also been reported that elevated expression of autophagy in cancer cells has been implicated in the development of PDAC (Rui Kang 2012).

PDAC is characterized by a dense stromal matrix also referred to as TME. This stomal matrix starts to evolve early around PanIN lesions. Moreover, in established carcinomas, the TME constitutes up to 90% of the tumor mass. PDAC TME is composed by various cellular and acellular components. Between the acellular components, large amounts of collagen, fibronectin, proteoglycans, and HA are present (Provenzano et al. 2012; Jacobetz et al. 2013). Particularly, HA is consistently increased in pancreatic cancer stroma, where exerts a pro-tumoral action (Sato et al. 2016), including tumor growth (Toole and Hascall 2002), cell proliferation, invasion (Cheng et al. 2016), and metastasis (Zhang et al. 1995; Toole 2002). These actions, associated with tumor progression, require that HA binds to its specific receptors. The main binding receptor, CD44 is located on the cell surface, and the intracellular RHAMM (receptor for hyaluronan-mediated motility) in the cytoplasm, where it activates the PI3K/Akt and ERK1/2 signaling pathways (Zhu et al. 2013; Lokeshwar et al. 2014). Moreover, CD44 has been recognized as a cancer stem cell marker of PDAC, involved in both epithelial mesenchymal transition and multidrug resistance; mechanisms involved in cancer cells protection from chemotherapeutic agents (Zhang et al. 2012; Wei et al. 2013). The secretion of HA by tumor cells facilitates the action of cancer-associated fibroblast. These cells produce growth factors, hormones, and cytokines to stimulate HA production by PDAC cells, which stimulates and promote their malignant behavior. As a result, the interaction between HA and its receptors provides tumor cells the suitable microenvironment to survive, proliferate, and invade. HA not only works as a tumor promoting factor, but also provides a barrier to the arrival of chemotherapeutic agents. In this sense, the accumulation of HA in TME increases the interstitial fluid pressure (IFP), which makes drug perfusion very difficult (Dufort et al. 2016a, b). It was described that hyaluronidase treatment can rapidly reduce the IFP in a mouse model of PDAC and significantly improve the appropriated response to gemcitabine (Provenzano et al. 2012). It is correct to think that reducing the level of HA in the ECM may offer an effective approach to treating PDAC.

It is attractive to presume that the inhibition of HAS individually with specific antibodies or the use of promoters of each hyaluronidase would be an adequate strategy to inhibit tumor progression and induce remission. However, the expression

of these enzymes in pancreatic cancer cells is heterogeneous (Cheng et al. 2016), suggesting that this strategy could not result in a successful response in most patients with PDAC. Rather, the use of compounds that reduce the synthesis of HA could be suitable as a strategy of HA reduction. Among them, 4-methylumbelliferone (4-MU), which stably suppresses the synthesis of HA in vitro and in vivo, has been widely accepted because of its mechanism of action and because it is innocuous to organisms (Kudo et al. 2017). Two therapeutic strategies were proposed: the combination of 4-MU with gemcitabine, the first-line chemotherapeutic agent used for decades, and decombination of 4-MU with immunotherapy. For the first option, it was described that 4-MU inhibited the formation enveloped HA-rich matrix, promoting the perfusion of gemcitabine (Nakazawa et al. 2006). In another study, in a xenograft mouse model, electron microscopic observation revealed that 4-MU reduced the amount of HA in tumors surrounding, on the backs of animals and altered the intercellular space (Yoshida et al. 2016). Moreover, 4-MU orally administrated, enhanced the survival rate of mice that were intraperitoneally inoculated with human PDAC cells. In these mice, intratumoral HA level was reduced in a 30% compared to control mice (Yoshida et al. 2016). This result is really encouraging, because it is supposed that orally administered 4-MU is distributed throughout the whole body, assuming that the beneficial effect would be not only on the primary tumor but also on distant metastases, causing suppression of proliferation, migration, and invasion activities.

The other therapeutic strategy involves the combination of 4-MU and immunotherapy. Within the last, immune checkpoint inhibitor therapy (targeting CTLA4, PD1, PDL1) or the perfusion of immune cells have not been remarkably successful against PDAC probably because of the strong and extensive ECM rich in HA. In this context, HA plays an integral role in this immune evasion. As explained above, the reduction of HA in the tumor ECM reduces the IFP, promoting drug perfusion as well as the arrival of immune cells. It was described that the administration in vivo of 4-MU reduced intratumoral HA level and promoted infiltration of inoculated γδ T-cells into tumor tissue, with the consequent suppression of tumor growth (Suto et al. 2019). However, further research is needed to achieve an adequate potential combined therapy. But it is promising that the control of HA will become an adjuvant treatment that can be used in combination with different immunotherapies, enhancing their efficacy through the remodeling of the ECM around cancer cells.

7.3.1.5 HA and Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cancer cause of death globally (Brenner et al. 2014). The global burden of CRC is projected to increase by 60% within 2030, especially in countries with medium and high human development index (HDI) scores (Wong et al. 2021). CRC originate primarily from genetic and epigenetic alterations in epithelial cells, leading to the development of benign polyposis, develops into adenoma and then carcinoma,

and finally the invasion of the interstitial matrix. Those processes require the degradation and modification of the existing ECM (Onfroy-Roy et al. 2020).

Several studies have shown the importance of HA in this type of cancer. HA levels are higher in cancer tissues than in noncancerous tissues. The stromal tissue stains highly intensely and the tumor epithelium is mostly HA negative, and this feature is associated with tumor grade, thus high-grade tumors have strong HA intensity. Furthermore, patients with strong HA intensity in tumor epithelium have a low survival rate (Wang et al. 1996; Ropponen et al. 1998). Also, cytosolic HA levels are correlated with clinical tumor stage, thus, patients with more advanced tumors have higher HA levels. However, HA content is not correlated with age, sex, tumor location, or histological grade (Llaneza et al. 2000). Zhang et al. evaluated the molecular weight (MW) of abnormally elevated HA in cancer tissues. HA MW was approximately 6 kDa and it was positively correlated with metastasis and invasion of CRC. Besides, 6-kDa HA was found elevated in sera from CRC patients and they observed a significantly positive correlation between 6-kDa HA expression in primary CRC lesions and matched serum samples from these patients (Zhang et al. 2019).

HA binds to CD44 that is expressed on the plasma membrane of many cells but also binds to other receptors including toll like receptor 4 (TLR4) that is widely distributed in the gastrointestinal tract. HA binding to TLR4 promotes epithelial repair in the DSS-colitis and radiation injury models (Riehl et al. 2012) and mediates the normal growth of the intestine and colon including the proliferation of must repair. b or p? epithelial stem cells (Riehl et al. 2015). However, HA–CD44–TLR4 interactions are involved in pathogenesis of CRC (Kim et al. 2004). Makkar et al. demonstrated that HA binding to TLR4 and CD44 promotes cell proliferation and blocks spontaneous apoptosis. In addition, TLR4 and CD44 knockdown or HA blocking by PEP1 slows tumor growth and reduces tumor volume (Makkar et al. 2019). Even more, the decrease in GAGs synthesis due to UDP-glucose dehydrogenase (UGDH) downregulation and 4-MU treatment reduce CRC cells growth and motility, indicating that invasive ability of CRC cells is HA dependent (Wang et al. 2010).

HA synthases (HAS) are also involved in the development and progression of CRC. The expression of HAS3 was known to produce smaller HA fragments with molecular size between 50 and 1000 kDa, as well as total HA production were found to be increased in metastatic CRC cells when compared with cells isolated from a primary tumor. Furthermore, downregulation of HAS3 in a highly tumorigenic CRC cell line decreased subcutaneous colon cancer growth in a mouse model by increasing apoptosis rate of these cells (Bullard et al. 2003). Even more, Kim observed a correlation between HAS2 levels and malignant phenotypes of CRC cells. HAS2 expression was significantly induced in CRC tissue samples and CRC cell lines. Furthermore, HAS2 depletion increased apoptosis, therapeutic sensitivity, and decreased epithelial to mesenchymal transition-related migration and invasive ability of CRC cells (Kim et al. 2019).

HA may influence metastasis of colon carcinoma cells. Laurich et al. demonstrated that metastatic colon carcinoma cells that express high levels of HAS and

synthesize large pericellular HA matrices adhere more avidly to laminin than cells isolated from a primary tumor that secrete and retain less HA (Laurich et al. 2004).

7.3.1.6 HA and Lung Cancer

Lung cancer is the leading cause of cancer death around the world. It arises from the cells of the respiratory epithelium and can be classified into two categories. Small cell lung cancer (SCLC) is a highly malignant tumor derived from cells exhibiting neuroendocrine characteristics and accounts for 15% of lung cancer cases and non-small cell lung cancer (NSCLC), which accounts for the remaining 85% of cases (dela Cruz et al. 2011). The pulmonary ECM provides mechanical stability and elasticity, which are essential for physiological lung function during the breathing, inhalation, and exhalation. Lung-ECM is mainly composed of collagens, proteoglycans, and glycoproteins and serves as a reservoir for several growth factors and cytokines, which are crucial for cell differentiation and proliferation. Lung tumors are surrounded by an extensive stroma and interactions between cancer cells-ECM protects cells from chemotherapy-induced apoptosis (Burgstaller et al. 2017).

The content of GAGs in cancer tissues is higher compared to normal tissues and the amount of these polysaccharides is different in every histologic type (Horai et al. 1981). HA concentration is increased mainly in squamous cell carcinoma, and it is related to the low survival of patients (Pirinen et al. 2001; Rangel et al. 2015). Moreover, sputum from lung cancer patients has higher HA concentration levels compared to cancer-free and healthy people (Rangel et al. 2015). Also, HA receptors are related to the development of lung cancer. RHAMM is upregulated in non-small cell lung carcinomas, especially in metastatic tumors (Wang et al. 2016) and there is an increase in LYVE-1 levels in tumor tissue and periphery related to lymph nodes metastasis (Renyi-Vamos et al. 2005).

HA is produced by tumor cells and stroma cells and several studies have shown the importance of HA in the progression of lung cancer. Song et al. demonstrated that conditioned medium of NSCLC cells increases HA production by lung cancer-associated fibroblasts (LCAFs) and the inhibition of HA production by these cells reduces tumor cells growth and proliferation. Even more, simultaneous silencing of HAS2 and HAS3 and CD44/RHAMM silencing reduced HA production and in vitro proliferation of NSCLC cells (Song et al. 2019).

On the other hand, HA production is regulated by cytokines and growth factors like tumor necrosis factor alpha (TNF-α), tumor growth factor beta (TGF-β), and endothelial growth factor (EGF), produced by tumor cells, fibroblasts, and immune cells (Tammi et al. 2011). Mulshine et al. demonstrated that NSCLC cells treatment with TGF/IL enhance HA production by malignant cells and this increase is related to epithelial to mesenchymal-like transition in NSCLC cells (Mulshine et al. 2010).

7.3.2 HA and Sarcomas: Osteosarcoma, Chondrosarcoma, and Fibrosarcoma

Osteosarcoma (OS) is the most common primary bone tumor and responsible for considerable morbidity and mortality due to its high rates of pulmonary metastasis. The prognosis of OS patients has improved dramatically with the introduction of chemotherapy; however, cases with metastases or unresectable tumors still have a poor prognosis (Arai et al. 2011). Several studies have implicated HA-rich ECM on osteosarcoma progression (Nishida et al. 2005; Tofuku et al. 2006; Hosono et al. 2007). Tofuku et al. found that HA synthesized by HAS3 promoted proliferation, invasion and degradation of ECM, biological functions crucial for metastasis and showed that the inhibition of HA synthesis by 4-MU suppressed proliferation and invasion of LM8 cells in vitro (Tofuku et al. 2006). Nishida et al. showed that the selective inhibition of HAS-2 mRNA in MG-63 osteosarcoma cell line by antisense phosphorothioate oligonucleotides reduced HA production by these cells what disrupted the assembly of cell-associated matrices and, consequently, hindered cell proliferation, motility, and invasiveness (Nishida et al. 2005). In a later study, these same authors examined the effects of exogenously added HA oligosaccharides on tumorigenicity of murine osteosarcoma cells LM-8, highly metastatic in mice, and human osteoblastic osteosarcoma cells, MG-63 and proposed that these small oligosaccharides compete with the binding of high-molecular-mass HA for cell surface receptors such as CD44 as a mechanism to deplete HA-rich matrices from cells. They determined that the treatment of MG-63, which have abundant HA-rich cell-associated matrix, as well as LM-8, with HA oligos inhibited the formation of HA-rich cell-associated matrix, resulting in the inhibition of growth, motility, and invasiveness and induction of apoptotic activity in vitro. Even more, daily application of HA oligosaccharides showed a trend toward inhibition of LM-8 tumor growth in vivo. These data suggest that the abrogation of hyaluronan-rich cell-associated matrices have potent antitumor effects (Hosono et al. 2007). Similarly, Arai et al. showed that the inhibition of HA retention by 4-MU reduced the formation of functional cell-associated matrices in OS cells, and inhibited cell proliferation, migration, and invasion, resulting in the reduction of tumorigenicity and lung metastasis. Even more, although 4-MU showed only a mild inhibitory effect on the growth of the primary tumor in vivo, it markedly inhibited the development of lung metastasis by inhibiting HA retention (Arai et al. 2011). Even more, Gvozdenovic et al. showed that overexpression of CD44 in SaOS-2 cells enhances intratibial primary tumor growth and formation of pulmonary metastases using an intratibial xenograft OS mouse model. These malignancy-enhancing effect of CD44s was HA-dependent and was reflected in the significant increase of primary tumor volume and the numbers of pulmonary micro- and macrometastases when compared to that of control SaOS-2 cells. These results suggest that CD44s/HA interactions play a key role in OS (Gvozdenovic et al. 2013). Even more, it was shown that hyaluronidase increased the transcapillary pressure gradient in OS xenografts by degrading extracellular HA and remodeling ECM, thus improving the uptake and

distribution of liposomal doxorubicin (Eikenes et al. 2005). Taken together, these reports highlight the involvement of HA in the progression and metastasis of OS.

Chondrosarcoma, the second most common primary malignant bone tumor, particularly when low-grade, is also characterized by the formation of a HA-rich ECM which has been considered as one explanation of drug resistance (van Oosterwijk et al. 2012). In this sense, Hamada et al. showed that the inhibition of HA synthesis by 4-MU suppressed cell proliferation, migration, and invasiveness of chondrosarcoma cells. Even more, in an in vivo model of chondrosarcoma, the inhibition of HA production markedly inhibited grafted tumor growth (Hamada et al. 2018). Recently, Koike et al. evaluated the effects of a novel hyaluronidase, KIAA1199, on ECM formation as well as antitumor effects on chondrosarcoma. They demonstrated that the KIAA1199 forced expression did not affect proliferation or apoptosis but inhibited migration and invasion of RCS cells in vitro. In contrast, the expression of KIAA1199 significantly inhibited the growth of grafted tumors. Although there was no direct inhibitory effect on proliferation in vitro, induction of KIAA1199 showed the antitumor effects in grafted tumor growth in vivo possibly due to changes in the TME such as inhibition of ECM formation. Together, KIAA1199 could be a novel promising therapeutic tool for low-grade chondrosarcoma, mediated by the degradation of HA (Koike et al. 2020).

Other antecedents have also provided direct evidence for the role of HA in tumorigenicity in fibrosarcoma, another of the most common sarcomas. In this case, Kosaki et al. showed that overproduction of HA by expression of the HAS2 enhances growth of tumors in xenograft models (Kosaki et al. 1999).

7.3.3 HA and Glioblastoma

Glioblastoma (GBM), also known as grade IV astrocytoma by the World Health Organization classification (Louis et al. 2016), is the most frequent primary tumor of the central nervous system (CNS) in adults. It is characterized by fast growth, invasiveness, and high mortality, with a median survival of less than 15 months after diagnosis (Anjum et al. 2017). Currently, there are few therapeutic options (le Rhun et al. 2019; Perus et al. 2019; Strobel et al. 2019), with the first-line therapy being surgical resection and radiotherapy combined with cycles of temozolomide (TMZ). Unfortunately, TMZ therapy causes severe adverse effects, and almost 50% of patients exhibit resistance to the treatment (Anjum et al. 2017; Philteos et al. 2019; Strobel et al. 2019). Furthermore, high inter- and intratumor heterogeneity, individual variability and different stages of disease at diagnosis time complicate GBM treatment (Rajaratnam et al. 2020).

For several years there is a growing interest in studying the CNS's ECM and the mechanisms through which it impacts in the development and progression of brain tumors, with obvious implications for the development of new therapeutic alternatives. In the CNS, HA is the main component of ECM, beside with proteoglycans (such as aggrecan, versican, neurocan, and brevican without collagen), the tenascins

and link proteins, and other GAGs, including chondroitin sulfate, heparan sulfate, and keratan sulfate (Eikenes et al. 2005). In the CNS, HA participates in the correct generation, proliferation, and maturation of neural stem cell progenitors (NSCP) during brain development and repair (Su et al. 2019). Reports published in the 70s and 80s established that HA has another, undesirable role: these studies demonstrated that production of GAGs—and particularly HA—by malignant glioma cells was higher than those in normal glial cell lines (Pibuel et al. 2021b), and which was associated with a higher rate of cell proliferation and several publications did confirm a correlation between the addition of HA and an enhanced rate of invasion across multiple glioma cell lines (Chintala et al. 1996; Nakagawa et al. 1996; Radotra and McCormick 1997; Pibuel et al. 2021a).

After that, several approaches have been used in the study of the relationship between HA and GBM including in vitro 2D and 3D assays, in vivo models and patient samples (Pibuel et al. 2021b). In GBM, CD44 is strongly involved in cell invasion (Mooney et al. 2016), and it was demonstrated that optimal levels of this receptor were necessary for GBM cells to generate highly infiltrative tumors in a mouse model (Klank et al. 2017), while the treatment with an anti-CD44 monoclonal antibody inhibited tumor growth of local glioma (Breyer et al. 2000). Regarding RHAMM, it has been associated with an increase in migration and proliferation of glioma cells, and its levels have been correlated with tumor grade (Akiyama et al. 2001; Virga et al. 2017; Lim et al. 2017). Although the importance of CD44 and RHAMM in mediating HA's functions is clear, other receptors such as EGFR in association with the HA receptors could explain some of the effects of HA on GBM progression. It was demonstrated that CD44 through the bind of EGFR form complex, that might provide a mechanism for HA-mediated cell invasion and proliferation (Tsatas et al. 2002).

After these interactions, HA activates several signaling pathways being the most relevant MEK/ERK, c-MYC, PI3K/Akt, FAK, RhoA/ROK, and the transcription factor NF-κB (Pibuel et al. 2021b), enhancing all the malignant features of GBM, mostly proliferation, migration, and invasion but also chemoresistance (Kim et al. 2005). The activation of these signaling pathways increases the activity of MMPs, the CD44 cleavage and in turn an increase of migration and invasion of this tumor type (Chetty et al. 2012).

Regarding the tissue samples of patients, glioma lesion showed HA content much higher than in adult normal brains (Delpech et al. 1993); however, the proportion of HA was inversely associated with glioma grade (Sadeghi et al. 2003). Interestingly, although HA failed as a molecular marker, HAS2 and HYAL-2 were associated with tumor grade and even correlate with patient outcomes (Valkonen et al. 2018; Pibuel et al. 2021b).

Considering the poor outcomes of the patients with GBM, several studies focused its efforts on the development of new therapeutic alternatives and molecular targets. The increases of HA in tumor tissues compared to the healthy brain, discussed above, may not have obvious clear cut diagnostic potential, but they do implicate HA in GBM progression, the results suggest its potential as a therapeutic target. HA

degradation has been explored as a therapeutic target in several studies, and some clinical trials have shown that treatment with HYAL as adjuvant chemotherapy contributes to clinically relevant remissions in high-grade astrocytoma and to the efficacy of chemotherapy in pediatric brain tumors (Baumgartner et al. 1998; Martinez-Quintanilla et al. 2015). In addition, it was recently reported that treatment with HYAL, alone or in combination with TMZ, showed a cytotoxic effect on the GSC population (Hartheimer et al. 2019). Interesting results were also obtained with oHA. The oHA treatment improved the effects of both radiation and methotrexate and even TMZ on GBM cells (Maria et al. 2008; Karbownik et al. 2014). Finally, as a new alternative, the use of 4MU showed a marked effect on GBM cells decreasing metabolic activity, cell proliferation, migration, and MMP-2 activity while causing high levels of apoptosis in murine GL26 GBM cells. Moreover, our group showed that 4MU partially diminishes HA synthesis, although several of its effects would be independent of this inhibition (Pibuel et al. 2021a).

Due to this, it is one of the main targets for the investigation is to find alternative therapies that seek to control tumor spread and reduce mortality, one way is understanding the interaction of HA with GBM cells.

In this sense, the mechanical interplay between the recruited cell stroma, the ECM, and the tumor cells has therefore attracted significant attention as a new avenue of therapy, termed stromal therapy.

7.4 Conclusion and Perspectives

To sum up, we could conclude that the ECM component GAG molecule, hyaluronan has tumor specific characteristics, and is an important factor in the development and progression in different hematological and solid tumors. It is involved either in direct or indirect way in all process defined as the hallmarks of cancer (Fig. 7.2).

It is possible to conclude that, molecules involved in HA metabolism and signals could be considered as clinical targets for tumors beyond consideration of the etiology. However, for the clinical application, it is important to consider that the impact of its modulation effect depends on the type of tumor. Thus, its application could be associated with induction of tumor cell death, avoidance of drug resistance to the enhancement of immunotherapy. With this perspective, we have described and discussed the different aspects of the role of HA in the biology and therapy of various tumors in this chapter. Finally, we aimed to support help the strategies for pharmacological application able modulate HA and consequently different processes in cancer progression.

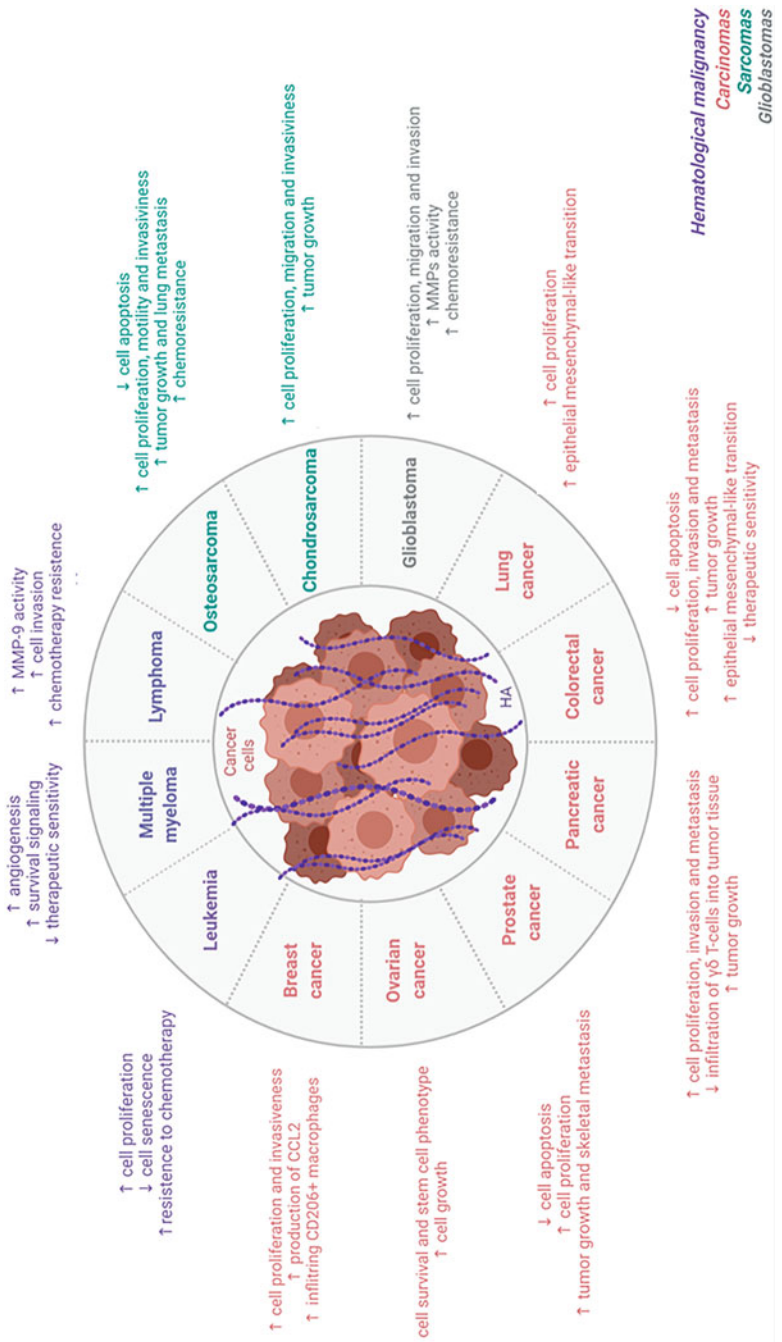

Fig. 7.2 Biological effects of ECM Hyaluronan (HA) in hematological malignancy and in carcinomas, sarcomas and gliomas

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Chapter 8
Heparanase: A Paramount Enzyme for Cancer Initiation, Progression, and Metastasis

Valentina Masola, Nicola Greco, Giovanni Gambaro, Marco Franchi, and Maurizio Onisto

Abstract Consolidated data indicate that tumor bulk is not only made up of a heterogeneous set of neoplastic cells but also of a variety of resident and infiltrating host cells, soluble factors, and components of the extracellular matrix which as a whole is defined as the tumor microenvironment. In this context, the extracellular matrix plays a fundamental role in tumor progression as it acts as a repository for various biomolecules such as growth factors, cytokines, enzymes, and inhibitors which are mainly linked to heparan sulfate proteoglycans (HSPG) and whose release can regulate the response or not of cancer cells. Among the various enzymes involved in the degradation of the ECM, heparanase (HPSE) has been shown to be particularly involved in tumor progression and metastatic invasion. This enzyme, capable of cutting heparan sulfate (HS) chains, is overexpressed in practically all solid tumors, clearly demonstrating that it has pro-invasive and pro-angiogenic characteristics for neoplastic cells.

Furthermore, considering that heparanase is released not only by tumor cells but also by platelets, endothelial cells, and immune cells, we can admit that its enzymatic activity has a strong impact on the tumor microenvironment. Here, we discuss the recent development in the study of heparanase in cancer progression as well as on novel mechanisms by which heparanase regulates the nature of the tumor microenvironment.

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8.1 Introduction

The life of almost all cells that make up the tissues of multicellular organisms is influenced by the surrounding microenvironment. The signals arriving at individual cells can be both physical and chemical in nature and can depend on contacts with adjacent cells and structural molecules or on soluble factors released by cells or tissues located nearby. This bidirectional communication between the microenvironment and the cells that are immersed in it therefore plays a fundamental role in tissue homeostasis both in normal and pathological conditions such as in the case of tumors (Quail and Joyce 2013). Tumor microenvironment (TME) has been defined as the environment containing the cancer cells and extracellular matrix (ECM). Beside the growing tumor includes blood vessels, fibroblasts, immune and inflammatory cells, signal molecules and the various structural molecules that make up the interstitial stroma (Baghban et al. 2020). The importance of the tumor microenvironment is demonstrated by the fact that evolving environmental conditions and signals from growing tumors continually induce changes in the composition of TME, increasingly underlining the need to develop new therapeutic strategies to target the microenvironment rather than the tumor itself (Labani-Motlagh et al. 2020).

For this reason, it is important to stress the concept that, in addition to the structural function, the tumor ECM can also acts as a storage reserve for numerous bioactive molecules, such as growth factors, cytokines, chemokines, and enzymes which bind mainly to the HS proteoglycans (HSPG) owing to the negative charges present on the GAGs (De Pasquale and Pavone 2020; Bartolini et al. 2020). The heparan sulfate proteoglycans (HSPG) constitute a heterogeneous group of molecules consisting of various types of proteins to which heparan sulfate (HS) linear chains of variable length are covalently linked. HS consists of the repetition of a disaccharide unit formed by a glucuronic acid (GlcA) or its iduronic acid epimer (IdoA) and by an *N*-acetyl glucosamine (GlcNAc) or *N*-sulfo glucosamine (GlcNS). The bond that joins the disaccharides is of type α (1 → 4) or β (1 → 4), while the interdisaccharide bonds are always α (1 → 4). HSPGs can be mainly present in the intracellular environment or on the cell membranes and in the extracellular space. HSPGs associated with cell surface are classified into two groups: syndecans (with transmembrane domain) and glypicans (which bind through glycosylfosfatidylinositol (GPI) anchors) (Karamanos et al. 2018, 2021). Membrane HSPGs can undergo a proteolytic cut of the protein core with consequent release from the cell surface (shedded ectodomain) or they can act as scaffold protein, coreceptor, endocytic, and adhesion receptors (Manon-Jensen et al. 2010). Pericellular HSPGs, on the other hand, refer those components of the extracellular matrix that are associated with the membrane of different cell types through integrins and/or cell surface receptors. This group of HSPGs includes perlecan, agrin, collagen XV and XVIII, the components of basement membranes. Perlecan is an HSPG that contains three HS chains at its N-terminus and which, in addition to its structural role, can be a storage tank for various growth factors including vascular endothelial growth factor

(VEGF), TGF-β, and FGF family members (Iozzo and Schaefer 2015). Agrin has an organization similar to perlecan with three side chains of HS. Together with laminin, collagen IV and nidogens, agrin, and perlecan are the fundamental constituents of the basement membrane (BM), which is the specialized laminar structure of the extracellular matrix that separates a connective tissue from a nonconnective tissue such as the epithelial layer (Iozzo 2005). Collagen XV and XVIII, having the structural properties of both a collagen and a proteoglycan, are other components of the basement membranes (Karamanos et al. 2021). In this scenario, the only enzyme capable of degrading the HS chains of HSPGs is heparanase, described and characterized since the 1980s by the group of I. Vlodavsky (Vlodavsky et al. 1999).

Heparanase-1 (HPSE) is an endo-β-D-glucuronidase, cutting heparan sulfate chain at the level of the β-1,4-glycosidic bond between glucuronic acid and glycosamine residue, generating 5–10 kDa fragments of HS. The human heparanase-1 gene is located on chromosome 4q21.3 and by alternative splicing express two mRNAs containing the same open reading frame (ORF) (Vlodavsky et al. 1999). In addition to HPSE-1, there is HPSE-2, a protein 40% similar to heparanase-1, which has no glycosidase activity but seems to act as inhibitor for HPSE-1 (McKenzie et al. 2000).

Heparanase is synthesized in the endoplasmic reticulum as a pre-HPSE precursor and has a very complex pathway of maturation.

The inactive pre-HPSE form is converted into pro-HPSE of approximately 68 kDa, through the proteolytic cleavage of the signal peptide located in the N-terminal region and subsequently processed in the Golgi apparatus. The precursor is then internalized in vesicles to be secreted into the extracellular space where it interacts with many membrane-bound molecules including mannose 6-phosphate and low-density lipoprotein receptors as well as syndecan-1 (Levy-Adam et al. 2003; Simizu et al. 2004; Ben-Zaken et al. 2008). From the extracellular space, it is carried inside the cell by endocytosis. In the lysosome, the pro-HPSE form undergoes a proteolytic cut by the enzyme cathepsin-L which leads to the formation of two active peptides (Abboud-Jarrous et al. 2008).

Once the crystalline structure of human HPSE-1 has been determined, it has been shown that this enzyme includes a TIM-barrel domain where the catalytic site is located, and a C-terminal domain essential for secretion and for the regulation of its enzymatic and nonenzymatic activity (Wu et al. 2015).

As far as protein expression is concerned, in healthy tissues and in physiological conditions, HPSE shows very low levels of expression and is limited to a few cell types such as keratinocytes, trophoblast, platelets, mast cells, and leukocytes. Conversely, in pathological conditions such as tumor progression and metastasis, inflammation, EMT, and fibrosis, HPSE is overexpressed. By means of immunehistochemical analysis, in situ hybridization, and real-time PCR, it has been demonstrated that heparanase is upregulated essentially in almost all human tumors examined (Masola et al. 2020; Vlodavsky et al. 2012).

Since more recent studies have shown that heparanase is not only produced by tumor cells but also by endothelial cells and by activated immune cells, there is more and more evidence that its activity has a strong impact on the tumor

microenvironment by promoting growth, neo-angiogenesis, and formation of a metastatic niche. In confirmation of this hypothesis, it has been shown that the overexpression of heparanase in transgenic mice (Hpa-Tg) makes the tumor microenvironment more favorable to the development of tumors in various experimental animal models (Boyango et al. 2014). Collectively, these results led to the hypothesis that heparanase plays a fundamental role in promoting the early stages of tumorigenesis.

The present chapter intends to summarize all the concepts and experimental evidences so far described concerning the involvement of HPSE in the initiation, progression, and metastatic process of cancer cells in relation to the tumor microenvironment.

8.2 Heparanase, Tumor Invasiveness, and Metastasis

Tumor invasion and metastasis can be defined as the result of a series of steps of interaction between the neoplastic cells and the microenvironment that surrounds them, and which implies their ability to modify the scaffolding of the tissues through the degradation or remodeling of the molecular structures that form and give rise to the extracellular matrix (Hanahan and Coussens 2012; Hinshaw and Shevde 2019). These invasive and metastatic capacities of tumor cells constitute one if not the main hallmark of malignant tumors compared to "in situ" tumors and are the cause of the majority of cancer-related deaths. It is now a consolidated fact that for this function, cancer cells have at their disposal a battery of degradative enzymes capable of "cutting" all the various macromolecules that make up the ECM surrounding the neoplastic mass, and whose overexpression correlates directly with the tumor progression and the formation of metastases. Among these degradative enzymes, the various matrix metalloproteases (MMPs), aspartic and cysteine proteases, together with the urokinase-type Plasminogen Activator (uPA) should be mentioned (Gialeli et al. 2011; Stetler-Stevenson and Yu 2001; McMahon and Kwaan 2015). In cooperation with these proteolytic enzymes, heparanase acts and contributes to tumor progression thanks to its ability to cut HS chains, thus altering the structure and function of HSPG and consequently contributing in a fundamental way both to the remodeling of the extracellular matrix and to the cleavage of syndecans and glypicans bound to the cell surface. From an experimental point of view, several studies have confirmed the role of HPSE in promoting tumor invasiveness by demonstrating how the overexpression of this enzyme increased the metastatic potential of different tumor cell lines, and correlated with reduced survival in various tumor xenograft models (Vlodavsky et al. 1999; Ilan et al. 2006). As counter-proof of this role, by inhibiting the activity of HPSE by means of gene silencing or specific inhibitors, a reduction in the invasive capacity and metastasis of various tumor cell lines was obtained both in vivo and in vitro (Edovitsky et al. 2004; Masola et al. 2009). A further confirmation of HPSE as a promoter of tumor invasiveness was obtained by the immunohistochemical analysis of tumor samples which then

highlighted how the higher HPSE positivity was attributable to the areas of tumor invasiveness, while, on the contrary, the adjacent healthy tissue showed no detectable signal level (Beckhove et al. 2005; Tang et al. 2002).

It should also be borne in mind that, in particular for tumors of epithelial origin, the possibility of invading adjacent tissues requires the tumor cell to lose its adhesion with other tumor cells and to change shape by acquiring mesenchymal cell traits. This epithelial to mesenchymal transition (EMT) process, originally described during gastrulation in embryonic development, was subsequently observed and described in various pathological events including tissue fibrosis, tumor invasiveness, and metastasis (Di Gregorio et al. 2020; Dongre and Weinberg 2019). During EMT, epithelial cells tend to lose cell–cell adhesion junctions and apical–basal polarity and acquire a mesenchymal phenotype with a marked propensity for motility (Kalluri 2009). Various experimental studies on different animal models of tumors strongly support the concept that EMT is a key mechanism in tumor progression aimed at invasion and metastasis (Dongre and Weinberg 2019). In this context, the role played by heparanase in promoting EMT has been well documented in both renal fibrosis and tumor pathology (Masola et al. 2016; Li et al. 2016). Bearing in mind that among the major inducers of EMT there are both FGF and TGF-β and that these factors are present in the extracellular space mainly linked to the HS chains of proteoglycans, the action of heparanase allows the cutting of the HS chains and consequently the release of these factors which can then act on the tumor cells inducing the EMT (Masola et al. 2012, 2014; Pang et al. 2016).

Finally, it should be mentioned that a fundamental step of the metastatic cascade is the process of intravasation and extravasation of invasive tumor cells that enter the circulatory system directly or through the lymphatic network (Wong and Hynes 2006). These steps therefore imply the ability of tumor cells to degrade the ECM and more particularly the subendothelial basement membrane (BM). BM is a particular form of ECM in which, in addition to laminin, type IV collagen, and nidogen, there are also three typical HSPGs such as agrin, perlecan, and collagen XVIII (Iozzo and Schaefer 2015). In concert with the role played by MMPs (in particular, MMP-2 and MMP-9 specifically for collagen IV), the activity of HPSE toward the HS chains contributes decisively to regulating the passage of tumor cells through the BM in the metastatic process (Shuman Moss et al. 2012; Reiland et al. 2004). This hypothesis has been increasingly confirmed over the years by several follow-up clinical studies in which patients with high HPSE expression showed increased tumor angiogenesis, a higher incidence of metastases, and a worse prognosis than patients with low or no HPSE expression. Furthermore, in experimental xenograft models, it has been shown that nude mice inoculated with HPSE-silenced tumor cells show a longer survival, a lower rate of liver infiltration/metastasis, and a lower rate of neo-angiogenesis with respect to controls (Vlodavsky et al. 2012). Overall, all of this experimental and clinical evidence allows us to affirm that HPSE is, for all intents and purposes, a key player in the process of invasiveness and metastasis of tumors.

8.3 Heparanase and Tumor Angiogenesis

The process of neo-angiogenesis, which is the formation of a new network of blood vessels, is fundamental to supply the tumor cells with nutrients and oxygen, to allow the growth of the neoplasm and to initiate metastatic dissemination. The enzymatic activity of heparanase toward HS chains contributes significantly to neo-angiogenesis because, as previously described, agrin, perlecan, and collagen XVIII HSPGs are structural components of the subendothelial basement membranes of blood capillaries and, therefore, their degradation allows endothelial cells to proliferate and migrate in response to angiogenic stimuli. In all this, the action that heparanase can carry out is twofold and synergistic: on the one hand, the cutting of the HS chains involves the remodeling of the ECM but, on the other hand, it determines the release and diffusion of growth factors and molecules linked to the ECM, such as VEGF-A, FGF-2 which are potent pro-angiogenic factors (Ilan et al. 2006; Vlodavsky et al. 1996). One of the first confirmations of the pro-angiogenic role of HPSE was obtained from the analysis of transgenic mice in which the relative overexpression of HPSE was accompanied by increased vascularization. In particular, in the mammary glands of hpse-tg mice, there was an excessive branching and grouping of alveoli connected to an interruption of the basement membrane and to a greater vascularization. The hpa-tg mice also showed an accelerated rate of hair growth, correlated with high expression of heparanase in hair follicle keratinocytes and increased vascularity (Zcharia et al. 2004). To confirm these effects on the neo-angiogenesis process, it was then demonstrated with immunohistochemical analysis that a preferential staining of the HPSE protein was evident in the endothelium of the capillaries and small germinative vessels, while the mature quiescent blood vessels did not show any positivity (Elkin et al. 2001).

In 2016, a new HPSE-dependent mechanism for inducing neo-angiogenesis was unveiled in myeloma cells. In these tumor cells, most of the pro-tumorigenic effects of HPSE reside in the ability to increase expression and shedding of syndecan-1, a proteoglycan which is critical for the growth of myeloma cells (Ramani et al. 2013). Specifically, shortening the HS chains on syndecan-1 HPSE provides greater access to the core protein of syndecan-1 for metalloproteinase 9 (MMP9), thus facilitating cleavage of syndecan-1. The extracellular cleavage of syndecan-1 allows its release from the cell surface thus uncovering a cryptic domain which is an unveiled site for both the VEGF-2 receptor and the α4β1 integrin present on the cell membrane of both myeloma cells and endothelial cells. This coupling of the receptor with the integrin leads to the activation of VEGF-2 receptor signaling which promotes augmented invasion and angiogenesis of myeloma. Roneparstat, an HPSE inhibitor, reduces the spread of syndean-1 and consequently inhibits tumor invasion and neo-angiogenesis (Jung et al. 2016).

Also on another experimental myeloma model, it has recently been shown that heparanase overexpression is associated with reduced levels of CXCL10, a member of the CXC chemokine family that binds to the CXCR3 receptor and is involved in the induction of apoptosis, chemotaxis and which suppresses the growth of myeloma

by attenuating the proliferation of endothelial cells and, therefore, neo-angiogenesis (Strieter et al. 2006). Since the expression of heparanase is inversely proportional to the expression of CXCL10, this experimental evidence highlights a new mechanism that allows this enzyme to control tumor angiogenesis in myeloma (Barash et al. 2014).

8.4 Heparanase and Tumor Microenvironment

ECM represents a reservoir for bioactive molecules and it provides a strong support to tumor growth and metastasis (Folkman et al. 1988). Heparan sulfate is an essential element in cell signaling and communication. HS has the ability to bind growth factors and cytokines and thereby create a reservoir of signaling molecules in the ECM and on the surface of cells (Bartolini et al. 2020; Iozzo and Sanderson 2011). HS controls growth-factor activity in the microenvironment by regulating the half-life of the ligand, its diffusion, and its interaction with tyrosine kinase receptors (Casu et al. 2010). ECM changes modulate the activation of intracellular signaling pathways (Kim et al. 2011), causing downstream variations in gene expression (Fattet et al. 2020). Consequently, it regulates cellular adhesion, cytoskeletal dynamics, cell invasion and migration, cell proliferation and survival, differentiation, stem cell-like phenotype, as well as epithelial–mesenchymal transition (EMT), cell metabolism, and chemosensitivity (Cox 2021). Cell surface HS proteoglycans such as syndecans, especially syndecan-1, are actively involved in tumor onset and cancer progression. In particular, syndecans are shedded by proteases (Fears and Woods 2006) and the subsequent action of heparanase changes their physiological roles (Piperigkou et al. 2016). For instance, it has been shown that HPSE-mediated shedding of syndecan-1 liberates peptide fragments with VEGF receptor activity, and this sustains angiogenesis (Jung et al. 2016).

Therefore, HS remodeling by HPSE has important implications for remodeling of the cellular microenvironment during inflammation and cancer progression (Iozzo and Sanderson 2011).

Several works have proved that HPSE is expressed not only by tumor cells but also by cells of the tumor microenvironment (stroma, fibroblasts, and inflammatory cells) (Vlodavsky et al. 2012; Weissmann et al. 2016; Hammond et al. 2014). Thus, targeting the tumor microenvironment by heparanase inhibitors enhances the antitumor activity of approved therapies (Ritchie et al. 2011; Noseda and Barbieri 2020). For instance, in the bone marrow microenvironment, heparanase regulates the retention and proliferation of hematopoietic progenitor cells (Spiegel et al. 2008), modulates clonogenicity, proliferative potential, and migration of mesenchymal stem cells (Cheng et al. 2014), as well as shifting the differentiation potential of osteoblast progenitors within the myeloma bone microenvironment from osteoblastogenesis to adipogenesis (Ruan et al. 2013). Inflammatory cells are a key component of the tumor microenvironment, and they exert many activities promoting metastatic dissemination and metastatic niche formation. Since

heparanase is involved in both leukocyte and macrophage activation/migration, it is therefore no surprise that there is a tight relation among heparanase, inflammation, and cancer microenvironment (Higashi et al. 2020; Elkin 2020).

Heparanase also contributes to the formation of metastases-regulating exosomes in the tumor microenvironment (Thompson et al. 2013). Exosomes mediate tumor–host cell interactions both locally and at metastatic sites (Peinado et al. 2017). It has been proved that the increased amount of HPSE in the tumor microenvironment greatly increases exosome secretion through modulation of syndecan-1 signaling (Thompson et al. 2013; Baietti et al. 2012). In addition, heparanase is present on exosome surfaces (Sanderson et al. 2019), and this is one of the mechanisms by means of which heparanase induces chemoresistance in myeloma (Purushothaman and Sanderson 2020).

In summary, heparanase expressed by tumor cells and by the cells of the tumor microenvironment regulates the aggressiveness of cancer, and represents an important contributor to bad outcomes among cancer patients.

8.5 Heparanase, Autophagy, and Evading Cell Death

Autophagy is an evolutionarily conserved catabolic pathway required to remove unfolded proteins and damaged organelles, and thus maintain cellular homeostasis. In cancer cells, however, autophagy is further induced by starvation and stress, sustaining their high metabolic rate and promoting cell survival (Levy et al. 2017). Recent data suggest that heparanase enhances tumor growth and chemoresistance by promoting autophagy (Shteingauz et al. 2015). The mechanism by means of which heparanase regulates autophagy has not been completely described but it involves mTOR1 (Shteingauz et al. 2015).

Another mechanism by means of which heparanase may contribute to cancer cells evading cell death is the inhibition of apoptosis, indeed it has been documented that HPSE inhibition induces massive expression of cleaved caspase-3 in xenografts of medulloblastoma (Spyrou et al. 2017).

8.6 Heparanase in Inflammation

The tumor microenvironment is characterized by chronic inflammation and tumors have been described as wounds that never heal (Singel and Segal 2016). Numerous immune cells have been shown to be intimately involved with the tumor microenvironment (TME). While it is true that some infiltrating immune cells function in eliminating tumors, on the other hand, there exists a pool of immune cells that promote tumor growth and progression. Indeed, inflammatory cells persisting in the tumor establish a cross-talk with tumor cells that may result in a phenotype switch

into tumor-supporting cells (Dehne et al. 2017; Marzagalli et al. 2019; Gajewski et al. 2013).

The heparan sulfate proteoglycans (HSPG) plays an important role in cancer inflammation. Heparan sulfate (HS) is known to control inflammatory responses at multiple levels, including sequestration of cytokines/chemokines in the extracellular matrix (Jayatilleke and Hulett 2020; Xie and Li 2019), modulation of leukocyte interactions with endothelium and ECM (Higashi et al. 2020), and initiation of innate immune response through interactions with toll-like receptors 4 (TLR4) (Elkin 2020; Goodall et al. 2014). As a consequence, heparanase through the cleavage of HS of ECM may affect several aspects of inflammatory reactions, such as the regulation of pro-inflammatory immune-cell migration and activation, establishing acute and chronic inflammation, regulating secretion of cytokines and chemokines anchored within the ECM, and promoting lymphoangiogenesis (Higashi et al. 2020; Giese et al. 2019; Vlodavsky et al. 2016; Jayatilleke and Hulett 2020).

Leukocyte migration is characterized by adhesive interactions with endothelial cells leading to arrest, adhesion strengthening, crawling, and the migration of cells through the vessel wall and into inflammation sites (Higashi et al. 2020). The adhesion of leukocytes to the endothelial wall is facilitated by HS and other cell surface molecules (i.e., selectin and integrin), leading to cell arrest and the initiation of infiltration (Higashi et al. 2020; Collins and Troeberg 2019). A number of pro-inflammatory chemokines bind to HS, and this chemokine presentation plays a critical role in leukocyte recruitment. The enzymatic activity of HPSE produces a gradient of HS-bound pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-10, and TNF-α that stimulates the recruitment of leukocytes (Collins and Troeberg 2019; Xie and Li 2019). Studies on HS degradation have shown that HPSE is overexpressed in different types of innate immune cells such as neutrophils (Higashi et al. 2020), macrophages (Elkin 2020), DCs (Poon et al. 2014), and mast cells that mediate both acute and chronic inflammatory responses.

Neutrophils are the prevalent type of innate immune cells that are involved in acute inflammation. In model organisms like zebrafish, it has been demonstrated that neutrophils are often the first cells to arrive at sites of developing inflammation both at a wound, and during the early initiation phases of carcinogenesis. Neutrophils are the major mediators of acute inflammation and related tissue injury (Dehne et al. 2017). For example, HPSE expression has been shown to increase the expression of pancreatic cytokines (TNF-α, IL-6, etc.) and signaling molecule (phospho-STAT3) activity, along with enhanced edema and inflammation marked by neutrophil infiltration, which ultimately leads to acute pancreatitis (Khamaysi et al. 2017). In addition, studies on the mouse model of sepsis-associated inflammatory lung disease and heparanase null-mice suggest that HPSE activity induces neutrophil infiltration into pulmonary microvascular endothelial cells (Schmidt et al. 2012). In an interesting contrast, the enzymatic activity of HPSE may produce an anti-inflammatory effect on neutrophils, probably by disrupting the chemokine gradients with consequent impairment of recruitment (Massena et al. 2010). When in acute inflammation, the action of neutrophiles is not sufficient to completely resolve the inflammatory state, the infiltrating leukocytes changes from neutrophils to macrophages, which are

the major players in chronic inflammation. A large proportion of tumor-infiltrating immune cells are tumor-associated macrophages (TAMs), which are key promoters of inflammation and contribute strongly to cancer progression (Szebeni et al. 2017; Nathan and Ding 2010).

HPSE participates in macrophage activation resulting in increased production of pro-inflammatory cytokines (Elkin 2020). Macrophage activation is mediated by toll-like receptor (TLRs) activation through the soluble HS fragments produced by HPSE (Goodall et al. 2014). HPSE has been implicated in a number of macrophages-induced HPSE expressions in the colonic epithelial cells, promoting inflammation that drives the progression of colitis to colon cancer enhancing cancer progression (Putz et al. 2017). Macrophages interact with endothelial cells in an HPSE-mediated manner to maintain a chronic inflammatory condition, which aids the formation of a tumor-promoting microenvironment with NF-κB signaling and induction of STAT3 expression (Waterman et al. 2007). HPSE has been shown to generate a vicious cycle by stimulating macrophages, which induce the production and activation of epithelial–HPSE via TNF-α and cathepsin-L (Lerner et al. 2011; Menzel et al. 2006). HPSE expression has also been shown to promote macrophage activation, leading to TNF-α production in macrophages as well as in renal tissue, and to enhancement of chronic inflammation associated with diabetic nephropathy (Goldberg et al. 2014). Using a genetic approach, mice lacking HPSE have been shown to possess macrophages that express lower levels of cytokines such as TNFα, IL-1β, IL-6, and IL-10. Macrophages lacking HPSE show impaired phagocytic activity and reduced infiltrative capacity (Waterman et al. 2007).

Natural killer (NK) lymphocytes are lymphoid cells characterized by potent cytolytic activity against bacterial infection and tumors. In vivo experiment on a HPSE-deficient mouse has proved that the lack of HPSE in NK reduced their capacity to infiltration (Putz et al. 2017). On the other hand, it seems that the HS fragment, produced by HPSE activity, can interfere with NK-cell toxicity receptors (NCR) and thus with NK activation (Mayes et al. 2017).

Interestingly, HPSE plays a relevant role in CAR therapy. In fact, the data suggest that expression of HPSE in long-term ex vivo-expanded T cells coexpressing a tumor-specific CAR (chimeric antigen receptor) improves their capacity to degrade the ECM. Thus, HPSE can improve CAR-T antitumor activity without compromising their viability, expansion, or effector function (Caruana et al. 2015).

8.7 Heparanase, Exosome, and Chemoresistance

High expression of HPSE is strongly associated with tumor aggressiveness, greater tumor growth, angiogenesis, and metastasis with poor prognosis. It was demonstrated that exogenous HPSE induces tumor cells to increase exosome secretion, regulates their composition, and promotes tumor progression (Salem et al. 2016; Thompson et al. 2013).

Exosoms are membrane-derived particles belonging to the extracellular vesicles (EVs) and involved in signaling processes and intercellular cross-talk. They are released into the extracellular space from different cells in normal conditions but their secretion is highly upregulated in diseases (Mustonen et al. 2021) or tumors (Vlodavsky et al. 2020; Zahavi et al. 2021). EVs have been generally classified in three groups based on their mechanism of biogenesis and mainly considering their size: (a) exosomes, ranging from 30 to 100 nm in diameter, (b) microvesicles or microparticles, measuring from 100 to 1000 nm, and (c) larger apoptotic bodies (1–5 μm) (Rilla et al. 2014). EVs and also exosomes have been found in extracellular fluid filtrates like plasma or bile (Witek et al. 2009), breast milk (Admyre et al. 2007), saliva (Ogawa et al. 2008), urine (Pisitkun et al. 2004), semen (Brody et al. 1983), blood (Johnstone et al. 1987), ascites (Andre et al. 2002), cerebrospinal fluid (Vella et al. 2008), and synovial fluid (Mustonen et al. 2016). Recently, EVs and in particular exosomes were found in extracellular matrix (ECM) of most of connective tissues (Arasu et al. 2017).

Exosomes which derive from the luminal membranes of the endosome play crucial roles in mediating local and systemic cell communication (Salem et al. 2016). Exosomes are able to transfer their cargo including nucleic acids as microRNA and mRNA, lipids, proteins and signaling molecules to target cells and ECM in distant sites within the body. They may act as intercellular messengers or carriers of regulatory molecules into target cells, thus providing cell-to-cell distant communications, but they are also able to transfer important information from cells to the surrounding microenvironment (Salem et al. 2016; Rilla et al. 2014; Arasu et al. 2017; Madhusoodanan 2019).

Secretion and shedding of EVs into the surrounding ECM originates from both the apical and basal surfaces of the epithelial cells. EVs secretion represents a way to reverse intracellular content or molecules into the ECM. For instance, cells release Hyaluronan (HA) in extracellular spaces by shedding HA-coated EVs from the tip of short cytoplasmic filopodia or microvilli (Mustonen et al. 2016; Rilla et al. 2013). It is of interest that an increase of HA expression is related to an increased aggressiveness of cancers, so that EVs are evaluated as clinical biomarkers (Rilla et al. 2013, 2014; Properzi et al. 2013) or future therapeutics (Alvarez-Erviti et al. 2011; Kordelas et al. 2014).

Even though the modality and regulation of exosome uptake by target cells is still under discussion, it was fascinatingly suggested that filopodia could be involved in an exosome uptake which resembles the virus enveloping: exosomes were observed to surf along the filopodia surface to the body cell surface (Heusermann et al. 2016). Here, exosomes seem to entry into recipient cells by two main mechanisms: endocytosis and membrane fusion (Raposo and Stoorvogel 2013; Turturici et al. 2014; James 2016). It was suggested that exosome uptake is HSPGs-dependent, following the same mode previously described for several viruses. These proteoglycans are sorted to and associate with exosomes and act as internalizing receptors of cancer cell-derived exosomes or receptors of exosome cargo (Christianson et al. 2013).

When cancer cells develop a more aggressive phenotype, the secretion of exosomes radically increases. To confirm this data, it was reported that also high

amounts of enzymatically inactive forms of HPSE do not evidently increase exosome secretion (Thompson et al. 2013). Exosomes might be able to modify the composition of tumor microenvironment, directly digesting ECM components or indirectly controlling target cells in synthesizing or even degrading ECM molecules. In fact, exosomes are so small that they can easily penetrate into the depth of collagen meshwork of ECM, invade endothelial cells, and travel to lymph nodes and distant tissues to promote cancer metastasis (Conigliaro and Cicchini 2019). Exosomes interact with tumor microenvironment. Degradation of peritumoral ECM is supported by some proteinases which have been described in exosomes: matrix metalloproteinases (MMPs) are able to digest both basement membranes and other ECM molecules, but also aggrecanase which specifically degrade the aggrecan-rich ECM in some tumors and diseases (Arasu et al. 2017; Dolo et al. 1999). In exosomes is present also another enzyme: heparanase the sole heparan sulfate degrading endoglycosidase (Thompson et al. 2013; Vlodavsky et al. 2016). Both exogenous heparanase, which cleaves HS chains on proteoglycans in ECM, or also its increased expression develop exosomes production and secretion (Thompson et al. 2013). An increased expression of heparanase observed in many cancers including ovarian, pancreatic, myeloma, colon, bladder, brain, prostate, breast, liver tumors, and rhabdomyosarcoma represents a clinical marker associated to a high risk of metastasis and poor prognosis.

Heparanase can also affect the exosome protein cargo, which shows higher content of syndecan-1, VEGF, and HGF in exosomes secreted by heparanase-high expressing cells (Thompson et al. 2013; Baietti et al. 2012). In particular, it has been reported that HPSE trims long heparan sulfate chains into shorter ones and favors syndecans complexes that, by recruiting syntenin-1 (a cytoplasmic adaptor of HSPGs) and ALIX–ESCRT (endosomal-sorting complex required for transport machinery), improve endosomal intraluminal budding and production of exosomes (Baietti et al. 2012; Friand et al. 2015).

Intercellular communications between cancer cells and circulating lymphocytes is also suggested, as circulating lymphocytes in breast cancer patients can secrete heparan sulfate syndecan-1 which upregulating heparanase expression, and exosomes secretion could be a fundamental regulator of molecular mechanisms in carcinogenesis (Roucourt et al. 2015; Theodoro et al. 2019), together with syndecan-2 which can affect breast carcinoma progression through a regulation of cytoskeleton, cell adhesion, and invasion (Lim et al. 2015).

It was demonstrated that exosomes released by cells expressing high levels of HSPE favor spreading of cancer cells on fibronectin and promote intravasation much more than exosomes secreted by heparanase-low cells (Thompson et al. 2013). Data reporting that the effect of heparanase/exosomes is related to fibronectin, which in its fibrillar form seems to act as a scaffold favoring cancers cell invasion (Erdogan et al. 2017), and the fact that a dramatic syndecan-1 expression in stromal fibroblasts of invasive breast carcinoma is associated with an aligned ECM fiber array to favor cancer cell invasion (Yang and Friedl 2016) suggest that any HSPE/exosomes effect could be mediated by the other ECM components. Moreover, monoclonal antibodies which are able to neutralize HSPE can inhibit myeloma and lymphoma tumor

growth, and dissemination is the result of a combined effect on tumor cells and cells of the tumor microenvironment (Vlodavsky et al. 2016).

Anticancer drugs can induce therapy resistance by promoting the development of autophagy (Larrue et al. 2016; Sanderson et al. 2017) and although intracellular HSPE is primarily located in lysosomes, late endosomes, it is also found in autophagosomes (Coombe and Gandhi 2019). Heparanase enhances tumor growth and chemoresistance by favoring both exosomes secretion and inducing autophagy (Shteingauz et al. 2015). Interesting results suggest that exosomes interplay with tumor cells, and acting as barriers to anticancer therapy, play a dramatic role in supporting tumor chemoresistance (Zhang and Grizzle 2014; Au Yeung et al. 2016). Consequently, heparanase favoring the secretion and uptaking of exosomes and inducing autophagy promotes tumor progression and chemoresistance by affecting both tumor and host cells behavior (Shteingauz et al. 2015).

When human myeloma cells are exposed to chemotherapy, the increase of HSPE expression promotes exosomes secretion and enhances their association together in cells which survive chemotherapy. Exosomes induced by anticancer drugs, also called chemoexosomes, allow an easy degradation of heparan sulfate within ECM as they exhibit a different protease profile compared the untreated cells and an increase of HSPE on their surface. Metastatic breast cancer cells showing a relation between exosomes secretion and invadopodia development suggest that both ECM degradation and invasion finally depend on exosome MT1-MMP and other proteases (Hoshino et al. 2013).

Chemoexosomes trapped within the tumor microenvironment can immediately induce changing in neighboring tumor cells by increasing signaling pathways (e.g., ERK, P38) that promote chemoresistance. For instance, chemoexosome HSPG can be readily transferred to macrophages which improve their migration but also secretion of TNF-α, an important myeloma growth factor (Bandari et al. 2018). Chemoexosomes can also be stored within tumor ECM to exert their action on local ECM or migrate from the tumor microenvironment to distant tissues to develop premetastatic niches only later (Bandari et al. 2018; Peinado et al. 2011; Becker et al. 2016).

To confirm the crucial role of HPSE in regulating exosomes secretion and chemoresistance, some authors recently reported that in multiple myeloma cells overexpressing EVs carrying miR-1252-5p, a consistent reduction in the levels of HSPE occurred. This data suggest that this microRNA may contribute to negative regulation of HSPE expression and can represent a novel therapeutic approach to sensitize multiple myeloma cells to bortezomib treatment (Rodrigues-Junior et al. 2021).

8.8 Concluding Remarks

Along with cardiovascular diseases, cancer constitutes a major cause of death among people in high-income countries. Despite the numerous therapeutic approaches developed so far, the complete recovery of the patient is often hindered and impeded by the complexity and variability of the disease considering not only the molecular changes within the tumor cell itself but the alterations that occur within the tumor microenvironment.

In particular, what makes malignant tumors so difficult to eradicate both surgically and with radio-chemotherapy is their ability to invade surrounding tissues and metastasize, to promote angiogenesis, to regulate inflammation, and to evade the immune response and apoptosis. In all these aspects of tumor pathology, heparanase plays a fundamental role both at the level of neoplastic cells and also at the level of the tumor microenvironment where its neutralization seems to be sufficient to slow down tumor growth as demonstrated in some lymphoma models. Consequently, a possible pharmacological strategy should be aimed at the inactivation of HSPE both within the tumor mass and in the tumor microenvironment where the use of the heparanase inhibitors has proved particularly decisive in the control of minimal residual disease.

Several HPSE inhibitors are already in clinical trials as anticancer drugs and, for some, a certain therapeutic efficacy and good tolerability for the patient have already been proven. For the near future, we therefore hope that the development of new drugs aimed at inhibiting the activity of HPSE may have a better therapeutic value not only in the oncology field, but also for all the other pathologies for which its involvement has been amply demonstrated.

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Chapter 9
Laminins and Matrix Metalloproteinases Connection: A Subtle Relationship That Can Go Wrong in a Tumor Context, Particularly If CD44 Gets Involved

Patricia Rousselle and Konrad Beck

Abstract Laminins (LM) are large extracellular glycoproteins involved in several biological processes, including cellular interactions, self-polymerization, and binding with other extracellular matrix proteins. LMs influence cell function by inducing various signaling pathways via cell membrane receptors and have multiple, often cell type-specific, functions in, for example, adhesion, differentiation, migration, and phenotype maintenance, and they also provide resistance to apoptosis. They are also important components of basement membranes. The basement membrane is partly degraded in the course of tumor growth, facilitating the invasion of budding cells and their migration to lymphatic or blood vessels. In this context, LMs undergo proteolytic cleavage, which disrupts their involvement in maintaining the structural and biological properties of the basement membrane. LMs are also involved via their participation in cancer cell adhesion and migration processes. These events are either supported by their major cell binding domains or triggered by cryptic interaction sites revealed by matrix metalloproteinase (MMP)-induced proteolytic cleavage. While being ideal targets for MMPs, LM can enhance their expression and activity. They appear to be key matrix elements in the regulation of MMP activity via the recruitment of the CD44 receptor, a multiple MMP-interacting and activating platform playing an important role in cancer progression.

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Abbreviations

ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
FAK	Focal adhesion kinase
GAG	Glycosaminoglycan
HA	Hyaluronic acid (hyaluronan)
HBS	Heparin binding site
HS	Heparan sulfate
LG	Laminin (C-terminal α-chain) globular domain
LM	Laminin
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
PI3K	Phosphatidylinositol-3-kinase

9.1 The Laminin Protein Family

Laminins (LMs) are a family of glycoproteins found in basement membranes with biological and structural properties that attract a growing interest. As essential structural constituents of the extracellular matrix (ECM), LMs also maintain close relationships with cells and transmit important morphogenetic information (Miner and Yurchenco 2004) (Domogatskaya et al. 2012). All LMs are composed of three different gene products, termed α, β, and γ chains, which are assembled into a cross-shaped heterotrimer αβγ. The three chains assemble within the endoplasmic reticulum via their C-terminal regions to form a triple stranded α-helical coiled coil rod and are covalently linked by disulfide bonds at both extremities of the coiled coil (Beck et al. 1993) (Matsui et al. 1995). The primary structures of five α chains (α1, α2, α3, α4, and α5), four β chains (β1, β2, β3, and β4), and three γ chains (γ1, γ2, and γ3) were identified by cDNA sequencing, leading to at least 18 heterotrimeric isoforms (Table 9.1, Fig. 9.1). Some genes encoding LMs may produce more than one subunit isoform due to alternative splicing; short-chain and long-chain splice variants are annotated with A and B, respectively (Aumailley et al. 2005). All basement membranes contain at least one LM isoform, and some may have two or three different isoforms. The expression and assembly of the various subunits vary in a spatio-temporal manner, which suggests biological and structural roles specific to each isoform (Yap et al. 2019). Some isoforms appear very early in embryogenesis and are markers for a given ECM, whereas others appear later and in a tissue-specific manner. In general, expression of the α5 subunit is the most widespread, whereas that of the α1 subunit is the most restricted (Miner et al. 1997). The α2 subunit is particularly abundant in tissues of mesodermal origin (cardiac and skeletal muscle), and the α4 subunit is abundant in endothelial and mesenchymal cells.

Table 9.1 Laminin isoforms

Name	Chain assembly	Previous	Original name
Laminin-111	α1β1γ1	1	EHS laminin
Laminin-211	α2β1γ1	2	Merosin
Laminin-121	α1β2γ1	3	S-laminin
Laminin-221	α2β2γ1	4	S-merosin
Laminin-3A32	α3Aβ3γ2	5 or 5A	Kalinin, epilegrin, nicein, ladsin
Laminin-3B32	α3Bβ3γ2	5B	
Laminin-3A11	α3Aβ1γ1	6 or 6A	K-laminin
Laminin-3A21	α3Aβ2γ1	7 or 7A	KS-laminin
Laminin-411	α4β1γ1	8	
Laminin-421	α4β2γ1	9	
Laminin-511	α5β1γ1	10	*Drosophila*-like laminin
Laminin-521	α5β2γ1	11	
Laminin-213	α2β1γ3	12	
Laminin-423	α4β2γ3	14	
Laminin-523	α5β2γ3	15	
Laminin-522[a]	α5β2γ2		
Laminin-212[b]	α2β1γ2		
Laminin-222[b]	α2β2γ2		
Laminin-333[c]	α3β3γ3		

[a]Laminin-522 reported so far solely in bone marrow
[b]The existence of either LM-212 or LM-222 is proposed based on studies of peripheral nerves in wild-type and LM α2 chain-deficient mice
[c]Laminin-333 is expressed at the apical specialization of adult rat testes, rather than in the testicular basement membrane

The α3 subunit, similar to β3 and γ2, is concentrated in epithelial basement membranes. The β1 and β2 subunits often have mutually exclusive expression, whereas the γ1 chain is present in all basement membranes (Durbeej 2010). LMs are essential at various stages of development. Thus, most LM mutants are fatal to the embryo and have made it difficult to study their functions at later stages (Yao 2017).

Although LMs are differentially recognized by cellular receptors, they all share a molecular organization based on the repetition of structural modules, giving them a common molecular organizational pattern. LM-111, which was initially extracted from the Engelbreth–Holm–Swarm tumor, was the first LM described as an asymmetric cross when visualized by electron microscopy comprising one long arm and three short arms carrying globular domains linked by linear segments (Fig. 9.2a). Other LMs do not exhibit all of the short arms, as they include "truncated" subunits at the N-terminal ends (α3, α4, β3, and γ2) (Figs. 9.1 and 9.2b). The N-terminus of each full-length LM chain consists of cysteine-poor sequences and are named LN domain. The short-arm rod-like portions consist of tandem arrays of LE modules, which sequences are related to the epidermal growth factor but contain one additional C-terminal disulfide bond. Inner globular structures within the short arms are

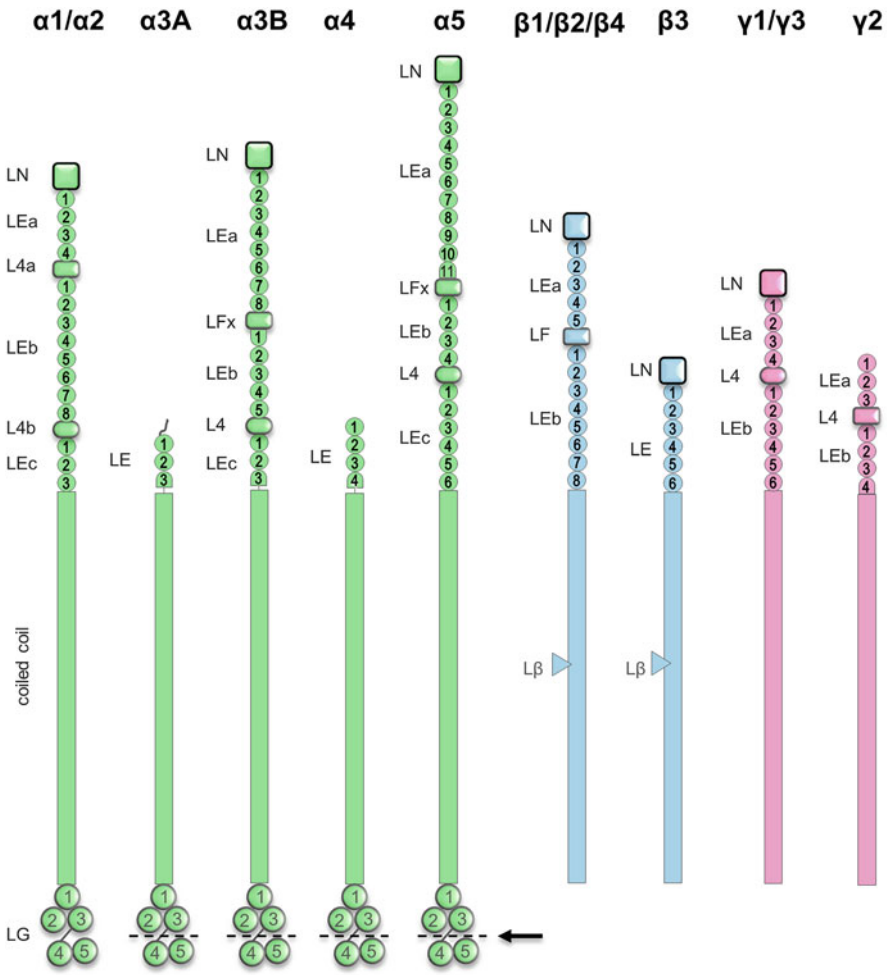

Fig. 9.1 Schematical models of the different laminin chains. LM chains contain tandem arrays of globular and rod-like regions. Chains with identical order of domains are combined (α1/2, β1/2/4, γ1/3). The N-terminal ends of all chains contain tandem arrays of a variable number of epidermal growth factor-like repeats LE with eight cysteine residues in short rod-like domains (LEa–LEc), as well as various globular domains (LN, L4, L4a, L4b, LFa, LFb, LFx), some of which can interact with integrins. Truncated LE domains containing less than 8 cysteines are depicted by half-circles. LE numbering follows the laminin nomenclature (Aumailley et al. 2005) though frequently, including in data base entries, they are counted consecutively starting at the N-terminus. The α chains have five globular domains (LG1–LG5) at the C-terminus. LG1–LG3 bind mostly to integrins, with some requiring the presence of the γ chain C-terminal region. LG4 and LG5 contain binding sites for dystroglycan (αDG), syndecans, and sulfated glycolipids. The LG4–LG5 tandem is thought to be cleaved off extracellularly (arrow) in α3, α4, and α5, though this is not the case for the α1 and α2 chains; LG domains of α2 stay attached to the molecule after cleavage. A small six cysteine residues containing domain Lβ of yet unknown biological function is present in the coiled coil regions of the β chains; originally the regions N- and C-terminal of Lβ were denoted by roman numerals II and I, respectively, but as this is unique to the β chains, this has been abolished. The LAMB4 gene coding for the β4 chain might be a pseudogene as no transcripts has yet been found

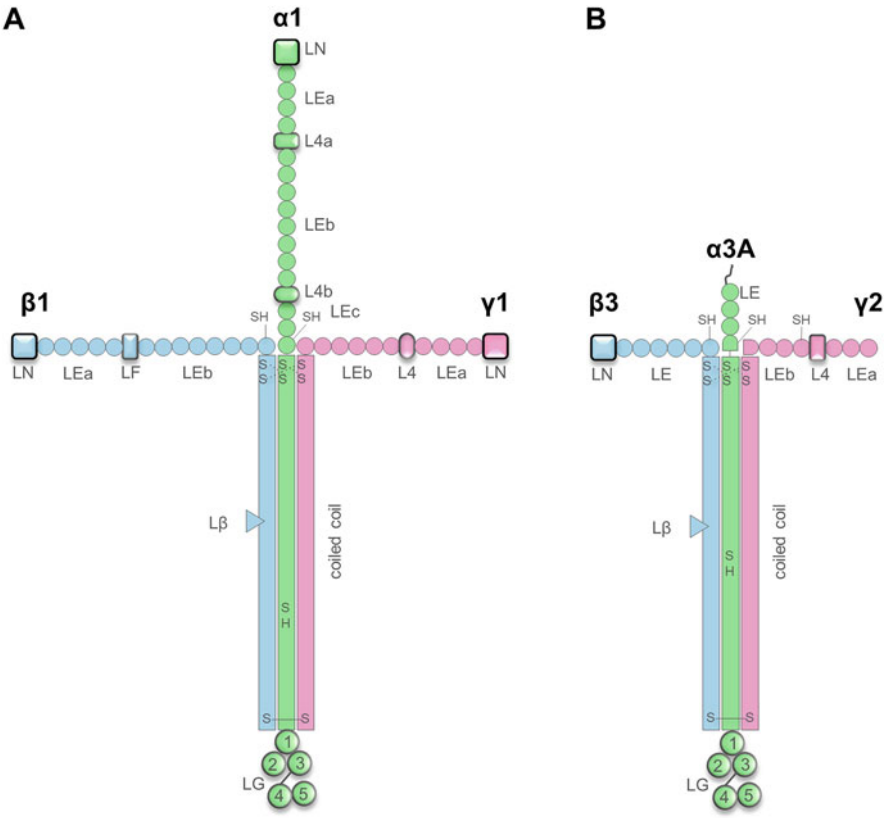

Fig. 9.2 Prototype assembly model of laminins. The α, β, and γ chains assemble to form a coiled coil in at least 18 combinations (Table 9.1). (**a**) LM-111 is a prototype heterotrimer able to self-assemble and form a network due to the presence of LN domains at the N-terminal ends of its three subunits. (**b**) LM-3A32 is a prototype heterotrimer unable to self-assemble as its α and β subunits are truncated. S-S at the termini of the coiled coil regions denote the presence of disulfide bridges. SH indicates the location of free cysteine residues

the L4 domains resulting from a long insert between cysteines 3 and 4 of the canonical 8-cysteine pattern of an LE domain. The LF domains have a distant structural similarity to L4 but are unique with respect to their cysteine pattern (Pulido et al. 2017). The LFx domains share similar sequences at the N- and C-termini with LF, but have further insertions of two internally similar sequence regions. C-terminal regions of the α, β, and γ chains assemble into a three-strand α-helical coiled coil forming the long arm (Macdonald et al. 2010).

The amino acids involved in this structure are organized in a heptad repeat in which the first and fourth residue are of hydrophobic, and the fifth and seventh frequently of charged character (Paulsson et al. 1985). Within the β chains, the coiled coil region is interrupted by domain Lβ consisting of 30–40 residues including 6 cysteines, which biological function is not yet known. All LM α chains possess

a large globule at the C-terminal end that consists of five similar domains (LG1–LG5), each containing approximately 200 residues (Timpl et al. 2000; Hohenester 2019). LG3 and LG4 are separated by a flexible hinge providing access for proteolytical attack. All LMs are glycosylated, which regulates their biological functions in addition to stabilizing them (Morita et al. 1985; Inamori et al. 2016).

9.2 Laminins as Multifunctional Elements Within Basement Membranes

Early molecular and in vivo studies in *Drosophila*, *C. elegans*, and mice support the idea that LMs are the basic building block for initiating basement membrane formation (McKee et al. 2007; Jayadev and Sherwood 2017). Most LMs can self-associate into polymeric sheet-like networks that are tightly associated with the cell surface through their LG domains (Yurchenco et al. 1992; Li et al. 2003). This polymerization process is reversible and depends on both the concentration and presence of divalent cations. This assembly model involves and absolutely requires the N-terminal domain LN of the α, β, and γ subunits (Cheng et al. 1997; Hohenester and Yurchenco 2013) (Fig. 9.2a). LM networks are noncovalent in nature and appear more dynamic than the cross-linked collagen IV networks described as being responsible for the mechanical strength of basement membranes. Thus, collagen IV recruitment and further basement membrane assembly appear to depend on LM self-assembly and its LG-mediated cell surface anchorage. LMs containing at least one truncated subunit, and therefore lacking one LN domain (i.e., the α3A, α4, and γ2 chains), are nonpolymerizing LMs and form other types of supramolecular arrays to integrate the basement membrane (Fig. 9.2b). The epithelial LM heterotrimers α3Aβ3γ2, α3Aβ1γ1, and α3Aβ2γ1 establish specific molecular interactions leading to the formation of anchoring structures characteristic of epithelial basement membranes (Champliaud et al. 1996; Rousselle and Beck 2013). The matrix integration of LMs comprising a truncated α chain assembled with the β1 and γ1 subunits, such as LM-3A11 and LM-411, may involve known interactions between the β1 and γ1 chains with agrin, nidogen (entactin), and perlecan.

LMs provide interaction sites for many other constituents including cell surface receptors. Some LM isoforms are modified extracellularly by proteolytic processing at the N- or C-terminal ends prior to binding cellular receptors or other matrix molecules (Tzu and Marinkovich 2008). Cellular receptors, such as integrins, syndecans, α-dystroglycan, Lutheran glycoprotein, or sulfated glycolipids, predominantly bind to the five LG domains at the C-terminal end of the α chains. The LG1–LG3 region contains the integrin binding domains, which often require the context of the LM heterotrimer and, most importantly, the C-terminal end of the γ chain to be fully effective (Deutzmann et al. 1990; Rousselle et al. 1995; Ido et al. 2004, 2007, 2008; Navdaev et al. 2008). The major LM-binding integrins are α3β1, α6β1, α7β1, and α6β4 (Nishiuchi et al. 2006). The LG1–LG3 region of the LM α5 chain also

binds to the Lutheran blood group antigen/basal cell adhesion molecule, a cell surface protein consisting of five immunoglobulin-like domains (Udani et al. 1998; Vainionpää et al. 2006; Kikkawa et al. 2007). The LG45 region contains binding sites for α-dystroglycan, syndecans, and sulfated glycolipids (Gee et al. 1993; Talts et al. 1999; Smirnov et al. 2002; Wizemann et al. 2003; Yamashita et al. 2004; Utani et al. 2003; Hozumi et al. 2009; Carulli et al. 2012; Suzuki et al. 2005). The N-terminal globular LN domains of the α1 (Colognato-Pyke et al. 1995) and α2 chains (Colognato et al. 1997) as well as LN (Nielsen and Yamada 2001) and L4 (Sasaki and Timpl 2001) of the α5 chain can bind to α1β1, α2β1, α3β1, and αVβ3 integrins.

Deregulated expression and involvement of LMs and their integrin receptors in tumor development and aggressiveness have been demonstrated in recent years. They will not be the subjects of this review as they have been exhaustively covered recently (Maltseva and Rodin 2018; Qin et al. 2017; Ramovs et al. 2017; Rousselle and Scoazec 2020). Instead, here we will focus on the link between LMs and matrix metalloproteinases (MMPs) and demonstrate how it can become toxic in cancer by amplifying the tumor process. We will also examine the current state of understanding the status of LMs as ligands for the CD44 receptor and seek for a potential link with MMP activity.

9.3 Laminins Are Involved in the Regulation of MMP Expression and Activity

MMPs are a family of secreted and membrane-bound zinc-dependent endopeptidases that have the capacity to degrade all components of the ECM. MMPs play central roles in morphogenesis, wound healing, tissue repair and remodeling in response to injury, and in the progression of diseases, such as inflammatory and neoplastic diseases (Nagase et al. 2006). The MMP family comprises 25 related but distinct vertebrate gene products, 24 of which are found in mammals and can be divided into six groups: collagenases (MMPs 1, 8, 13, 18), gelatinases (MMPs 2, 9), stromelysins (MMPs 3, 10, 11, 17), matrilysins (MMPs 7, 26), membrane-type MMPs (MT-MMPs; MMPs 14, 15, 16, 17, 24, 25), and other MMPs (MMPs 12, 19, 20). The expression of MMP genes is transcriptionally regulated by a variety of extracellular factors including cytokines, growth factors, and ECM proteins (Gaffney et al. 2015). A number of studies have revealed that LMs or their proteolytic fragments can be involved in the regulation of MMP expression and/or activity. These subtle regulations that occur in a physiological context can be amplified in pathological situations such as cancer, contributing to tumorigenesis and invasion (Karamanos et al. 2021).

Early studies revealed that an interaction between LM-111 and the 67-kDa LM receptor promotes MDA-MB231 breast carcinoma cell aggressiveness through the upregulation of MMP-14 and enhanced gelatinolytic activity of MMP-2 (Berno et al.

2005). Subsequent studies have shown that LM-111 can increase MMP activity in human neural stem cells (Sypecka et al. 2009). Later, activation of MMP-9 expression was associated with an α2β1 integrin-dependent migration process when the human cervical cancer SiHa cell line was cultured on an LM-111-coated surface (Maity et al. 2011). A mechanism involving focal adhesion kinase (FAK), integrin linked kinase, phosphatidylinositol-3-kinase (PI3K), and extracellular signal regulated kinase was followed by increased DNA-binding activity of NF-κB and Ap1 and subsequent stimulation of MMP-9 gene expression (Maity et al. 2011). A similar signaling pathway was revealed when human breast cancer MCF-7 cells were plated on an LM-111-coated surface (Pal et al. 2014). In this study, the concomitant downregulation of TIMP1, a negative regulator of MMP-9 activity, led to enhanced MMP-9 proteolytic activity. Another context in which a link between LM and MMPs was established is the kidney. The LM α2 chain was shown to contribute to pathogenesis in the Alport glomerular syndrome via activation of FAK on glomerular podocytes leading to the downstream activation of MMP-9, MMP-10, and MMP-12 gene expression (Delimont et al. 2014). A β1 chain fragment released through MMP-2 processing, LN–LE1-LE4, was shown to modulate the behavior of pluripotent stem cells and promote the epithelial–mesenchymal transition (EMT) in an α3β1-integrin-dependent fashion, whatever the fragment was delivered to the cells as a soluble or immobilized factor (Horejs et al. 2014). The β1 chain fragment triggered the downregulation of MMP-2 in human and mouse cells, as well as the upregulation of E-cadherin and MMP-9 in mouse cells during spontaneous stem cell differentiation.

The cryptic domains of LMs (i.e., hidden in full-length LMs and exposed upon proteolysis) can elicit biological responses and be involved in the regulation of MMPs. For example, peptides containing the sequence S_{2099}IKVAV, which is adjacent to the N-terminus the C-terminal LG1 domain of the LM α1 chain, have been shown to induce the expression of MMP-9 by monocytes/macrophages (Corcoran et al. 1995; Khan and Falcone 1997), whereas intact LM-111 does not (Khan and Falcone 2000). A mouse α5 LG1 domain-derived peptide was shown to induce macrophage and neutrophil chemotaxis both in vitro and in vivo and to enhance MMP-9 and MMP-14 activities (Adair-Kirk et al. 2003). Furthermore, human γ2 chain fragments and LG4-derived peptides from the α3, α4, and α5 chains exhibit strong chemotactic activity toward leukocytes, neutrophils, and monocytes (Mydel et al. 2008; Kenne et al. 2010; Senyürek et al. 2014). Whether these activities are associated with activation of MMP expression or activity is an interesting question that remains to be answered. Another study revealed that LM-332 potentiates human monocyte differentiation to tumor-associated macrophage-like cells secreting a high level of MMP-9 (Kamoshida et al. 2014). Upregulation of MMP-1 and MMP-9 expression was also reported at both the transcriptional and protein levels in epidermal keratinocytes upon treatment with recombinant C-terminal LG4 or LG45 domains of the α3 chain, an event that relies on an IL-1β autocrine loop through the mitogen-activated protein kinase (MAPK) pathway (Momota et al. 2005; Utani et al. 2003; Michopoulou et al. 2020). Several peptide sequences involved in this mechanism were identified as heparin binding sites (HBSs) and

characterized as ligands for the proteoglycan-type cell surface receptors, the syndecans (Utani et al. 2003; Carulli et al. 2012; Rousselle and Beck 2013). However, the role of LM was not limited to this transcriptional activation as the α3 LG45 domain participated in establishing the gelatinolytic activity of epithelial podosomes in migrating keratinocytes during wound healing (Michopoulou et al. 2020). We showed that the α3 LG45 domain triggers the proteolytic activity of MMP-9 and MMP-14 in epithelial podosomes via a mechanism involving the cellular receptor syndecan-1 (Michopoulou et al. 2020).

9.4 Laminins Are Cell Adhesion Ligands for CD44

The cell surface glycoprotein CD44, a hyaluronic acid (HA) receptor, plays a role in the regulation of cell–cell interactions, cell adhesion, cell growth, migration, and invasion (Knudson et al. 1996; Ponta et al. 2003). CD44 is an 85- to 200-kDa transmembrane glycoprotein ubiquitously expressed throughout the body and is detected in both normal and tumor cells. CD44 is encoded by a single gene with 20 exons but has many different isoforms (Fig. 9.3a). It is composed of a distal extracellular N-terminal domain, a stem region, a transmembrane domain, and an intracellular cytoplasmic C-terminal domain (Fig. 9.3b). The standard form CD44s is the shortest and most commonly expressed isoform. It results from expression of the constant exons 1–10, which are found in all isoforms. A number of splicing variants (CD44v) result from the insertion of variable exons in the extracellular region near the membrane, called the stem region.

There are ten variant exons expressed in mice (designated v1–v10), whereas humans express only variant exons v2–v10 due to the presence of an in-frame stop codon in exon v1 (Screaton et al. 1993). The N-terminal portion of the ectodomain is of a compact, ordered structure, stabilized by three disulfide bridges, that contains a HA binding region and glycosaminoglycan (GAG) chains (Fig. 9.4a). The stem region varies according to the expression of the splicing domains in the CD44v isoforms, and the transmembrane and intracellular domains both play important roles in CD44 functionality and connection to the actin cytoskeleton through ezrin/radixin/moesin (ERM) proteins (Tsukita et al. 1994; Gal et al. 2003; Medrano-González et al. 2021). Importantly, upregulation of CD44 is often closely associated with abnormal tumor cell behavior (e.g., proliferation, survival, migration/invasion, and chemoresistance) (Bourguignon 2019; Medrano-González et al. 2021).

Understanding the various interactions of CD44 on a molecular structural level that would allow for reliable predictions of binding sites is difficult. The complex post-translational modifications by N- and O-linked glycosylation greatly depend on cell types, developmental, and environmental conditions. Large chondroitin and dermatan sulfate GAG chains attached to the stem region in CD44s, and heparan sulfate chains found within the variable regions are highly flexible making high-resolution structural analysis challenging. Except for the most N-terminal *ca.*

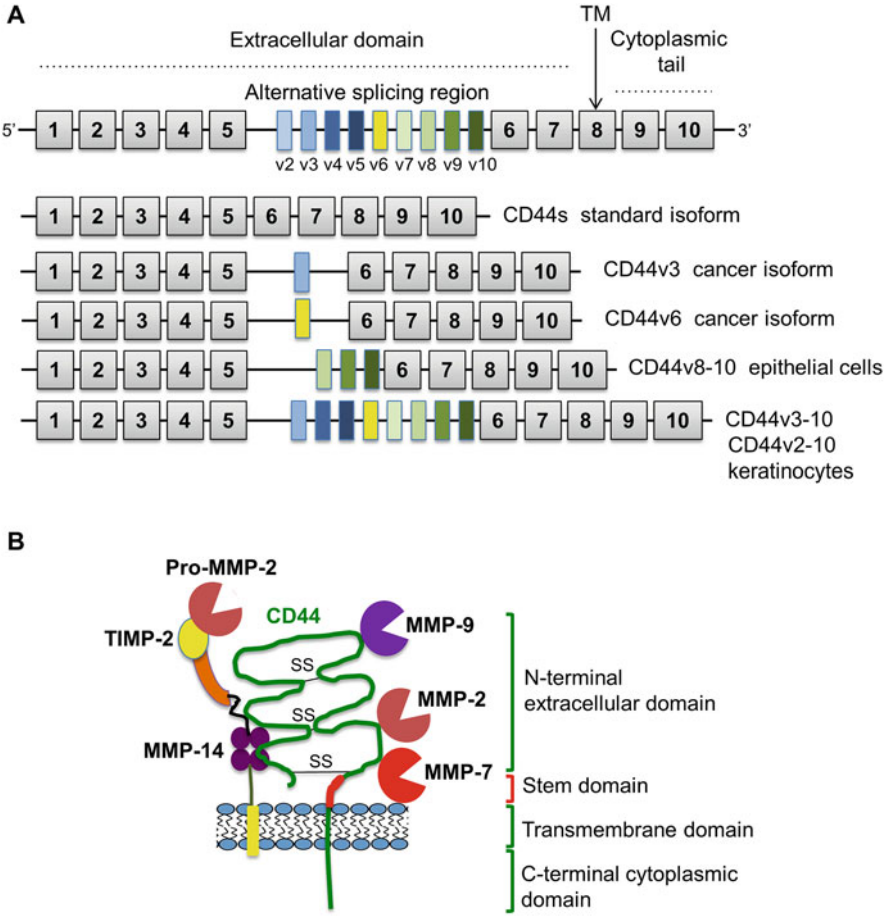

Fig. 9.3 CD44 as a platform for MMP binding. (**a**) Structure of the human CD44 gene, which consists of ten constant exons expressed in all CD44 mRNAs and proteins (grey boxes) and nine variant exons v (colored boxes) expressed in CD44 splice variants (CD44v). Other vertebrate genes contain an additional exon v1. The examples shown are the smallest isoform (standard CD44s, expressed ubiquitously in vertebrates) and the larger variant isoforms expressed in some epithelial cells and cancers. (**b**) CD44 protein structural domains. The CD44 protein is composed of an extracellular link domain (green), a stem domain (red), where the variant exon products are inserted, the transmembrane region, and the intracellular cytoplasmic domain. The CD44 extracellular domain is a platform for MMP-2, MMP-7, MMP-9, and MMP-14 binding and can indirectly activate MMP-2 through MMP-14 cleavage. Figure adapted from references Ponta et al. (2003); Cauwe et al. (2007); Medrano-González et al. (2021)

160 amino acids, even the unmodified ectodomain evades exploration by showing a high degree of intrinsic disorder with nearly 30% of residues being serine and threonine (Fig. 9.4b). In contrast to the classical view of structure–function relation as a lock-and-key system, the resulting flexibility on the structural level allows the

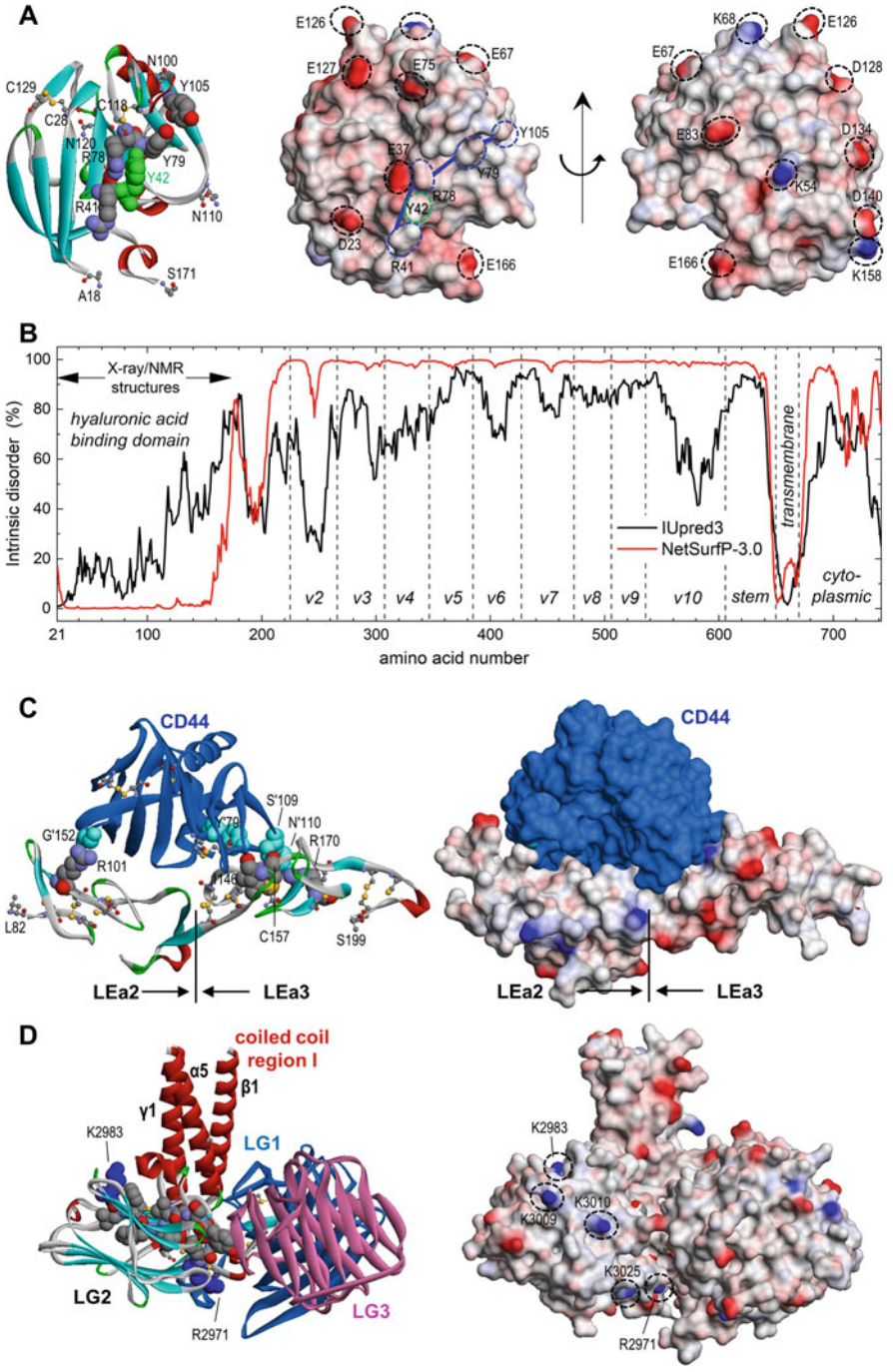

Fig. 9.4 Molecular constraints of CD44 and laminin interactions. (**a**) The protein backbone of the HA binding domain of human CD44 is shown on the left with α-helices in red and β-strands in cyan (PDB code: 4PZ4, ref. Liu and Finzel 2014). Terminal residues, disulfide bonds and putative

protein to adapt various conformations for optimal ligand binding (Habchi et al. 2014).

Even the various structures solved at high resolution by x-ray crystallography may not be regarded as static. Molecular dynamics simulations suggest that Y42 can act as a molecular switch that upon binding to HA changes the overall HA binding domain structure from a closed to open conformation that further increases affinity for HA (Guvench 2015).

CD44 is upregulated in a variety of cancers and can be expressed as CD44s or as a number of alternatively spliced CD44v isoforms. CD44 mediates its effects on the cancer cell by activating signaling pathways, such as protein kinases and transcription factors. The functional role of CD44 is pleiotropic, including induction of EMT and antiapoptosis, alterations in the cellular cytoskeleton, and promotion of drug resistance. CD44 is a compelling marker of cancer stem cells from many solid malignancies (Takaishi et al. 2009; Du et al. 2008; Zöller 2011). In addition, interactions between HA and CD44 promote epidermal growth factor receptor-mediated pathways, consequently leading to tumor cell growth, tumor cell migration, and chemotherapy resistance in solid cancers (Thapa and Wilson 2016; Yaghobi et al. 2021).

Fig. 9.4 (continued) acceptor sites for N-linked glycosylation are depicted as ball-and-sticks. Disulfide bonds follow a C1–C6, C2–C5, C3–C4 arrangement. Residues involved in HA binding are presented in CPK style as based on the similarity with the mouse sequence (R41, R78, Y79, Y105) (Banerji et al. 2007) Y42 (green) has been implicated in acting as a molecular switch inducing a conformational change upon interaction of R41 with HA to a higher affinity state (Guvench 2015). A solvent-accessible surface view of the same orientation is shown in the middle, and one obtained by rotation of 180° around the vertical axis on the right. The electrostatic potential is represented by red and blue for negative and positive charges, respectively. The position of various residues is indicated by dashed lines with those involved in HA binding highlighted and connected by blue lines. (**b**) Prediction of the intrinsic disorder of full-length human CD44 was performed using the IUpred3 (black line) (Dosztányi 2018) (https://iupred.elte.hu/) and NetSurfP-3.0 (red line) (Klausen et al. 2019) (https://biolib.com/dtu/nsp3) algorithms. (**c**) Putative interactions between the CD44 HA binding domain (blue) and LM γ2 LEa2–LEa3 domains are shown for the protein backbones on the left and the corresponding solvent-accessible surfaces on the right. Residues within hydrogen bonding distance between proteins are represented in CPK style, where the apostrophe denotes CD44 residues (R101-G′152, I146-Y′79, C157-S′109, R170-N′110). Simulations were performed using PatchDock (Schneidman-Duhovny et al. 2005) (https://bioinfo3d.cs.tau.ac.il/PatchDock/) and refined with FireDock (Andrusier et al. 2007) (https://bioinfo3d.cs.tau.ac.il/FireDock/). LM γ2 LEa2–LEa3 was modeled with SWISS-MODEL (Waterhouse et al. 2018) (https://swissmodel.expasy.org/) using PDB structure 4AQS of the mouse LM β1 LEa1–LEa4 as a template (Carafoli et al. 2012). (**d**) The protein backbone (left) and the corresponding solvent-accessible surface (right) of the C-terminal region of human LM 511 is shown (PDB code 5XAU, chains A, B, C) (Takizawa et al. 2017). The LM α5 sequence R_{2971}LVSYSGVLFFLK within the LG2 domain corresponding to the mouse peptide A5G27 (R_{2892}LVSYNGIIFFLK) shown to be involved in CD44 binding (Hibino et al. 2004) extends over one β-strand and is depicted in CPK style with the basic terminal residues highlighted in blue. The surface view indicates that further lysine residues (K3009, K3010, K3025) are arranged along a track that could facilitate interactions with GAG chains. Sequence numbering follows UniProtKB/Swiss-Prot data base entries P16070.3 (CD44), Q13753.2 (LM γ2) and O15230.8 (LM α5)

In addition to its direct signaling function, CD44 forms a platform at the cell membrane for the assembly of various MMPs with their substrates, ultimately to modulate cell migration (Cauwe et al. 2007) (Fig. 9.3b). CD44 is an important partner of MMP-9, playing a role in its anchorage to the cell membrane and preservation of its proteolytic activity (Yu and Stamenkovic 1999, 2000). Evidence suggests that the association of MMP-9 and CD44 in mouse and human tumor cells promotes invasion (Yu and Stamenkovic 1999; Gupta et al. 2013). Clustering of CD44 mediates MMP-9 relocation in breast tumor cells promoting its proteolytic activity on the membrane (Peng et al. 2007). The interaction of CD44 and the proteolytic form of MMP-9 is involved in the invasion activity of prostate cancer PC3 cells (Desai et al. 2007).

CD44 is also a molecular partner of MMP-14, playing a role in its translocation to the front of migrating cells (Mori et al. 2002). MMP-14 has been clearly characterized as a CD44 shedding enzyme with migration-promoting activity (Kajita et al. 2001). MMP-14 and CD44 are coexpressed at the lamellopodia of cells undergoing EMT (Cho et al. 2012). In normal mouse breast and uterine epithelium, the heparan sulfate (HS) chains of CD44v containing the exon v3 derived sequence bind active MMP-7 (Yu et al. 2002; Seiki 2002). CD44 also binds MMPs 2, 15, 16, 24, and 25 through their hemopexin domains in lamellipodia edges (Mori et al. 2002; Suenaga et al. 2005; Samanna et al. 2006) and can act indirectly by activating MMP-2 through MMP-14 cleavage (Zöller 2015) (Fig. 9.3b).

CD44 has been identified as a component of podosomes, which are protrusive adhesion structures involved in ECM degradation and mechanosensing (Murphy and Courtneidge 2011; Linder and Wiesner 2016). It was shown to colocalize with the F-actin core of podosomes in osteoclasts (Chabadel et al. 2007), human primary epidermal keratinocytes (Michopoulou et al. 2020), and in 3D-cultured macrophages (Van Goethem et al. 2011). By demonstrating a direct interaction between the LM α3 LG45 domain pair and the CD44 receptor expressed by human primary keratinocytes, we have revealed a determining role for this LM isoform in regulation of the proteolytic activity of MMP-14 and -9 in epithelial podosomes (Michopoulou et al. 2020). The 200-kDa CD44 isoform identified in our study is the long form v3–10 expressed by keratinocytes (Bourguignon and Bikle 2015). It is conceivable that the LM-332 LG45 domain concentrates active MMP-9 and -14 within epithelial podosomes through recruitment of CD44. In addition, the combination of LG45 and CD44 could facilitate binding of adaptor molecules to the cytoplasmic region in CD44 and activate multiple pathways involved in cell adhesion, migration, and proliferation including Ras, MAPK, and PI3K (Xu et al. 2020). Although this mechanism has been demonstrated in the physiological context of skin repair, it could undoubtedly be implemented in cancer cells in which the expression of LM-332 is frequently upregulated (Rousselle and Scoazec 2020). In cancer cells, the α3LG45 domain could regulate the expression and concentrate active MMP-9 and MMP-14 in invadopodia, the adhesive actin-rich membrane protrusions with high proteolytic activity found in cancer cells (Augoff et al. 2020). An argument in favor of this hypothesis is based on our study showing that Ras/IκBα-transformed keratinocytes lacking the LG45 domain exhibit a deficiency in MMP-9 and MMP-1

expression and a decreased invasive capacity (Tran et al. 2008). This deficiency was reversed by replacing LG45 through retroviral transduction. Whether the CD44 isoforms expressed in cancer cells can bind to α3LG45 is an important question (Chen et al. 2018). We already know that, in addition to the CD44v3-10 isoform, the 100-kDa standard CD44s expressed on melanoma A375 cells also binds the α3LG45 domain (Michopoulou et al. 2020; Takahashi et al. 1999), and we may wonder whether the various CD44 isoforms will bind to α3LG45 with different affinities. For example, the presence of variable exon 3, which encodes an HS chain carrying region, may impact the interaction (Bennett et al. 1995).

A link between LM-332 and the MMP-14/CD44 tandem was already revealed a few years earlier in a study showing that interactions between mature human thymocytes and LM-332 induces strong upregulation of active MMP-14 leading to CD44 cleavage and thymocyte migration (Vivinus-Nebot et al. 2004). As the LG45 domain was cleaved off and removed from the LM-332 used in this study, we can evaluate the possible participation of the N-terminal end of the γ2 chain, which also comprises an HBS, in this mechanism (Sasaki et al. 2001). One study revealed that the LN domain of the LM γ2 chain induces breast cancer MDA-MB-231 cell migration as a result of its binding to CD44 and phosphorylation of its cytoplasmic tail (Sato et al. 2015). Surprisingly, interaction studies uncovered a binding site for CD44 within the LEa2–LEa3 region, away from the HBS of the γ2 chain identified within the L4 and LEa domains (Sasaki et al. 2001; Ogawa et al. 2007), suggesting the involvement of a LM binding domain distinct from the GAGs in the CD44 ectodomain (Sato et al. 2015). However, the study did not mention any MMP-9 or MMP-14 recruitment, though it clearly reported a γ2 chain-dependent pro-migratory activation of CD44 reinforcing the hypothesis that LM-332 is a multisite ligand for CD44. Based on these data, we performed rigid body molecular docking simulations using a model of the LM γ2 LEa2–LEa3 pair and the coordinates of the HBS domain of CD44.The best result with a good global minimal energy conformation shows a good steric fit of CD44 within the groove of the LEa2-LEa3 connection and suggests four pairs of residues that come into hydrogen bonding distance (Fig. 9.4c). Though this model is in agreement with the experimental data, as any glycosylation and the stem portion of CD44s were not considered, it should be viewed with caution.

LMs were identified as ligands for CD44 already nearly 30 years ago. The binding of CD44 to a commercial LM preparation was detected in an ELISA-based assay using CD44 purified from lymphocytes (Jalkanen and Jalkanen 1992). The epithelial isoform CD44v8-10 (Brown et al. 1991), which is expressed in carcinoma cells such as human colorectal carcinoma KM-12, CCL 188, and MIP-101 cells, was identified as an LM-111 receptor (Ishii et al. 1993). These studies already reported that the interaction is mediated by the proteoglycan-rich region of the extracellular domain of CD44, an interaction site distinct from that of HA, which is located in the disulfide-rich N-terminus of the molecule (Ponta et al. 2003). In a study screening the adhesive properties of overlapping short peptides covering the entire LG1–LG5 domains of the α5 chain toward B16–F10 mouse melanoma cells, peptide A5G27 R_{2892}LVSYNGIIFFLK corresponding to one β strand of the LG2 module with the terminal basic residues in the surface exposed

loop regions was identified as a ligand for CD44 via its GAG moieties (Hibino et al. 2004). In the human sequence, this peptide relates to R_{2971}LVSYSGVLFFLK. When visualized within the solved terminal structure of LM 511 (Takizawa et al. 2017), it is evident that the basic side chains are surface exposed at opposite ends of the LG2 domain (Fig. 9.4d). Further positively charged surface areas are generated by K3009, K3010, and K3025. These five residues form a track that could further the alignment of a GAG chain. A similar track of basic residues crucial for heparin binding has been previously reported for the LM LG4 domain (Yamashita et al. 2004). The LM α5 chain interaction with CD44 stimulates melanoma cell growth, angiogenesis, migration, and metastasis (Hibino et al. 2005). CD44 overexpression in neural precursor cells significantly improved their spreading over LMs and increased the formation and length of filopodia, suggesting a role in migration (Deboux et al. 2013). Using a protein database approach, an important role for CD44 interactions with LM-111 was predicted in the axon growth of retinal ganglion cells during nerve regeneration (Ries et al. 2007). As CD44 is overexpressed on a wide variety of neuronal, epithelial, and hematopoietic malignancies, CD44 may be involved in either the malignant transformation of cells or the migration of malignant cells from the primary site to sites of metastasis. The mode of interaction of CD44 with LMs is very complex and can take place in a number of ways. Further work is now required to better understand these interactions and apprehend their biological consequences.

9.5 Laminins Are Targets of MMPs Activity

Like any other ECM protein, LMs are subject to total or partial proteolysis by MMPs, leading to the release of protein fragments, which may play a role in tumor progression. Over the years, MMPs have been found to play a remarkable number of regulatory roles at the cellular level, including pathways involved in apoptosis, immunity, cellular migration, and angiogenesis (Fingleton 2006; Itoh and Nagase 2002; Shay et al. 2015). Thus, MMPs play a major role in carcinogenesis. MMP functionality often complements classical tumor properties, leading to invasion, immune system avoidance, and metastasis (Winkler et al. 2020). The specific role of each MMP in cancer and their dysregulation in many cancer types have been accurately documented recently (Gobin et al. 2019; Piperigkou et al. 2021; Karamanos et al. 2021). An exhaustive gene expression study of 15 different types of cancers compared to normal tissue confirmed that MMP expression has a large degree of heterogeneity across cancers and revealed that the most prevalent gene expression changes were upregulation as opposed to downregulation in tumor tissue versus control tissue (Gobin et al. 2019). Facilitation of cell migration and invasion is thought to be the principal effect of the MMP-mediated breakdown of basement membranes surrounding cancer cells (Jayadev and Sherwood 2017; Hanahan and Weinberg 2011). All molecular networks, including LM networks, as well as ECM/cell adhesion complexes are targets of MMP-mediated proteolysis. Notably,

even if cells can physically breach basement membrane barriers in the absence of MMPs (Kelley et al. 2019), the massive production of MMPs by cancer cells greatly enhances their invasive capacity. Another barrier to tumor cell extravasation and invasion is the basement membrane underlying the endothelium of the vessel wall, where LM-411 and LM-511 are expressed (Spessotto et al. 2001). LMs can be cleaved by MMPs 2, 3, 7, 9, 10–16, 19, and 20 (Okada et al. 1986; Bejarano et al. 1988; Wilson and Matrisian 1996; Chandler et al. 1996; Ohuchi et al. 1997; Pirilä et al. 2003; Sadowski et al. 2005; Itoh and Seiki 2006; Laronha and Caldeira 2020), but invasive tumor cells primarily use MMP-2, MMP-9, and MMP-14 within invadopodia to cleave or degrade ECM proteins including LMs (Jacob and Prekeris 2015). Expression of MMP-14 in cancer cells correlates with their metastatic potential (Poincloux et al. 2009) and their ability to degrade fibrillar collagen and LMs. MMP-14 has the ability to activate MMP-2, MMP-9, and MMP-13 (Nishida et al. 2008; Knäuper et al. 2002; Li et al. 2017). The destruction of LM-111 by MMP-9 is thought to play a detrimental role in the early stage of breast cancer as the basement membrane no longer provides the appropriate signals to restrain epithelial cell proliferation (Beliveau et al. 2010).

The effects of LM degradation on cancer cell behavior, however, are more complex in the end because proteolytic cleavage leads to the release or exposure of either ECM-sequestered cytokines or a functional ECM fragment. Some LMs can express cryptic biological functions after proteolysis thereby altering cellular behavior and phenotypes (Niland and Eble 2021). The combined elastase/MMP-9-mediated proteolytic remodeling of LM-111 has been shown to unmask an epitope that triggers proliferation of dormant breast cancer cells through an α3β1 integrin signaling mechanism leading to aggressive lung metastases (Albrengues et al. 2018). This fascinating study demonstrated that the proteases present in neutrophil extracellular traps, together with DNA, are delivered to LM-111 through a DNA/LM interaction. LM-211, LM-411, and LM-511 could be degraded in the same way (Albrengues et al. 2018).

LM α1, β1, and γ1 chains are subject to MMP-2 and MMP-9 cleavage, releasing proteolytic fragments endowed with integrin- or syndecan-mediated cellular functions (Kikkawa et al. 2013). MMP-2 cleavage of the short arm of the β1 chain is predicted to disassemble the LM network, releasing a β1 LN–LE1-4 fragment that mediates α3β1-integrin-dependent embryonic stem cell adhesion (Horejs et al. 2014). Structural changes occur after LM-511 cleavage by MMP-9 (Gu et al. 2005), and fragments with activity toward inflammatory or cancer cells were identified within the LM α5 chain (Adair-Kirk et al. 2003; Hibino et al. 2004; Kusuma et al. 2011). An 80-kDa LM α2 C-terminal LG3–LG5 fragment possibly generated by MMP-9 is thought to play a role in reshaping the blood–testis barrier and spermatogenic function in a rat model (Gao et al. 2017). Analysis of the secretome from MDCK cells undergoing Ras-induced EMT resulted in the identification of two LM α5 internal and C-terminal fragments generated from MMP-1 cleavage with pro-migratory and pro-angiogenic activities (Gopal et al. 2016). LM α5 has also been reported to be cleaved by MMP-14, leading to prostate cancer cell migration (Bair et al. 2005).

LM-332 has been the focus of increasing interest over the last few years in the cancer research field, particularly the various proteolytic cleavages of its three subunits (Rousselle and Beck 2013). Many proteases degrade LM-332 and have been exhaustively documented (Rousselle and Scoazec 2020). To fulfill its essential function of anchoring proteins in epithelial basement membranes (Rousselle et al. 1991, 1997; Champliaud et al. 1996), LM-332 undergoes several physiological post-translational modification events. This maturation process allows its supramolecular integration and promotion of cell adhesion (Rousselle and Beck 2013). In cancers, additional cleavage events occur, compromising both these adhesive and connecting functions and additionally delivering pro-migratory signals to cells (Rousselle and Scoazec 2020). Cleavage of the β3 chain by MMP-7 or MMP-14 has been reported to enhance colon or prostate carcinoma cell migration and invasion, respectively (Remy et al. 2006; Udayakumar et al. 2003). MMP-2 or MMP-14 cleavage of the γ2 chain is reported to be a pro-tumorigenic signaling event (Giannelli et al. 1997; Koshikawa et al. 2000; Gilles et al. 2001). The released N-terminal LE repeats have been shown to induce EGF signaling and downstream MAPK activity in cancer cells (Koshikawa et al. 2004). The involvement of MMPs 3, 12, 13, 19, and 20 in the cleavage of the γ2 chain reinforces the status of LM-332 and its 2 subunit as an ideal target for MMPs in tumorigenesis (Pirilä et al. 2003; Sadowski et al. 2005; Väänänen et al. 2001).

Studies of the testis have reported that MMP-2 cleavage of the LM γ3 chain, part of the α3β3γ3 LM heterotrimer localized at the apical ectoplasmic specialization of Sertoli cells (Yan and Cheng 2006), induces dysfunction in the Sertoli cell tight junction permeability barrier through a 50-amino-acid residue peptide sequence in domain L4 (Siu and Cheng 2004; Yan et al. 2008; Su et al. 2012; Li et al. 2020). This process is thought to provide an efficient mechanism for modulating different aspects of spermatogenic function in response to changes in the epithelial cycle of spermatogenesis (Li et al. 2020). During zebrafish skeletal muscle development, LM was shown to act as an MMP-11 mediator and regulator of fibronectin levels at the myotendinous junction (Jenkins et al. 2016).

9.6 Conclusion

ECM remodeling is an important contributor to tumor progression and metastasis by contributing to the dissemination of cancer cells to distant tissues as well as angiogenesis. Among the many extracellular proteases expressed by human cells, MMPs are the most prominent enzymes supporting ECM proteolytic degradation and remodeling, as well as the modulation of cellular and ECM interactions. Whether MMPs are soluble/secreted in the tumor microenvironment or anchored to the cell membrane, their dysregulated expression in tumor cells, cancer-associated fibroblasts, and infiltrating immune cells is a hallmark of cancer. Being ideal targets of MMPs, the cleavage or degradation of LMs has structural and biological impacts favoring tumor development and metastasis. The fragments or peptides that are

generated can induce the production of more MMPs in various cell types, setting up a vicious cascade. The proteolytic activity of MMPs is often localized in adhesion structures, such as invadopodia, or occurs in proximity to the cell membrane where LMs are expressed. Cellular receptors, such as CD44, play an important role in addressing and regulating MMP activity. Their connection to LMs could promote the establishment of an active MMP-rich microenvironment prone to efficient degradation and remodeling of the ECM. Thus, LMs can be targets of MMPs, bioactive factors stimulating their expression, or even interacting platforms capable of recruiting and concentrating them.

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Chapter 10
Basement Membrane, Collagen, and Fibronectin: Physical Interactions with Cancer Cells

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Abstract Cancer cell invasion is regulated by extracellular matrix (ECM) chemical signaling and gene expression but it also consists in a mechanical process controlled by ECM's array. Scanning electron microscope analysis, invasion test, and real-time PCR demonstrated that Matrigel mimicking basement membrane (BM) doesn't promote epithelial–mesenchymal transition (EMT) in both low and very aggressive breast cancer cells (MCF-7 and MDA-MB-231). A loose network of type I collagen mimicking the sub-BM favors EMT in MCF-7 cells but physically limits their invasion ability vs. Matrigel, as collagen does not induce an increase of metalloproteases (MMPs) in cells following ameboid-invasion mode. Collagen doesn't change MDA-MB-231 phenotypes but further improves their invasion capability vs. Matrigel, by stimulating MMPs production. Concentrated type I collagen mimicking deeper ECM induces cells adhesion, further development of microvesicles, microvilli, long filopodia, and tunneling nanotubes (TNTs). Non-aligned fibronectin favors breast cancer cells adhesion, microvesicles, and TNTs development. Densely packed and parallel collagen fibers mimicking a collagen

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array in mammary tumor progression oppose invasion. In colon cancer, LoVo-R cells, resistant to doxorubicin, concentrated collagen favors development of invaginating phenotypes with invadopodia. We suggest that collagen acts as a physical factor inducing EMT in breast cancer cells and drug resistance in LoVo-R cells.

10.1 The Biomechanical Role of ECM in Cancer Progression

In normal conditions, extracellular matrix (ECM) connects but also separates different tissues and provides the gaseous and metabolic support to most of them. Similarly, progression of most diseases depends on the physical status and chemical properties of ECM which interacts and responds to the biological changes of the pathological processes. This is what usually happens in wounds, foreign body granulomas, as well as in cancer (Schäfer and Werner 2008; Fouad and Aanei 2017; Foster et al. 2018; Paolillo and Schinelli 2019).

Malignant transformation is related to an uncontrolled cells growth, dissociation of cells with loss of intercellular adhesiveness and acquisition of migration capability. These changes allow cells to breach the basement membrane (BM), invade the peri-tumoral microenvironment, and then colonize distant anatomical sites by forming metastatic points. In addition, the peri-tumoral ECM undergoes significant structural alterations which can also induce the epithelial–mesenchymal transition (EMT) in cancer cells (Zolota et al. 2021; Karamanos et al. 2021). As a matter of fact, the uncontrolled tumor growth generates by itself compressive stresses within tumor which can stimulate cancer cells migration by developing of invading leader cells and improving cell–substrate adhesion for grouped cell invasion (Tse et al. 2012).

Cancer cell invasion is foremost a mechanical process, and many experimental studies and reviews concerning the interactions between cancer cells and surrounding tissues have mainly focused on gene regulation and signaling that lead to the development of the tumor. However, the knowledge on the role that physical properties of tumor microenvironment exert in affecting cancer cell behavior is relatively limited (Brabek et al. 2010; Baker et al. 2011; Balkwill et al. 2012; Kim et al. 2018; Wang et al. 2018; Malandrino et al. 2018; Scott et al. 2019). In particular, changes in structural and biomechanical properties of ECM which favor cancer cell adhesion ability and development of new cell phenotypes with an improved motility just exerted by generations of mechanical actions like contractile and tensional forces are undervalued (Friedl and Brocker 2000; Ridley et al. 2003; Zaman et al. 2006; Mierke et al. 2008; Zanotelli et al. 2021). When cancer cells migrate and travel through the ECM, they encounter a mechanical resistance which cells must overcome in order to reach blood and lymphatic vessels.

From a chemo-mechanical point of view, ECM consists in a meshwork of insoluble proteins like collagen, assembling in collagen fibrils and then in fibers, and in minor amount elastin which forms the elastic fibers; but ECM also contains a

high molecular mass of anionic substances of predominant carbohydrate nature including proteoglycans (PGs) and glycosaminoglycans (GAGs), which are particularly able to sequester high amount of water. The insoluble collagen fibrils, mainly resisting to tensile stresses (Franchi et al. 2007, 2009, 2010; Pissarenko et al. 2019), are held together by GAGs and PGs which fill the interfiber spaces and acting like elastic strings in solution oppose compression. In ECM, these components interact with water and contribute to form a functional dynamic scaffold which can control the shape of tissues, organs, and whole body (Scott 1975, 2002, 2003; Yue 2014). ECM can bind cells of different tissues and offer anchorage sites for cell movement and migration, but also plays the role of a physical barrier separating different tissues or opposing to cell migration (Mould and Humphries 2004; Larsen et al. 2006; Lu et al. 2011; Yue 2014; Kular et al. 2014; Ringer et al. 2017).

ECM architecture has a significant effect on cell motility and the ability of cells to adhere and sense the mechano-chemical environment is particularly discussed. It is widely recognized that the chemical, mechanical, and topographical properties of the extracellular environment of peri-tumoral tissue can also play a possible primary role in affecting both tumor progression and cancer cells invasion (Lu et al. 2011; Baker et al. 2011). Collagen, the main component of ECM, is widely used as substrate in three-dimensional (3D) cultures of cancer cells. The chemical composition and physical characteristics of the ECM, such as geometry, macro-microporosity, collagen alignment, density, and stiffness, strongly contribute to cancer cell adhesion, migration, and invasion (Wolf and Friedl 2011). Moreover, both cancer and stromal cells can activate an enzymatic-dependent pericellular proteolysis which generates micro- and macrotracks delimited by relatively aligned collagen bundles which further favor cell adhesion and invasion (Gaggioli et al. 2007; Wolf et al. 2007; Provenzano et al. 2008).

Among other factors, also confinement and topology can affect modes of cell migration (Kurniawan et al. 2016; Pandya et al. 2017). Microporosity of ECM can depend on caliber, density, and array of collagen fibers, but also on the hydration level of tissue which is directly related to the content of GAGs like hyaluronic acid. Tissue mesh size, related to microporosity, represents a physical limit to cancer cell invasion and is related to deformation capability of the nucleus. Smaller than 7 μm^2 mesh sizes in ECM do not allow cancer cells to directly invade the peri-tumoral ECM (Wolf et al. 2013), whereas larger mesh sizes allow globular-shaped cells to migrate through the network by the so-called amoeboid movement. Even though collagen network plays a contrasting role to cancer cell invasion, some aggressive cancer cells can degrade the matrix architecture by releasing proteolytic enzymes named metalloproteases (MMPs) (Wolf et al. 2007; Friedl and Wolf 2010; Brabek et al. 2010; Scott et al. 2019).

Alterations of ECM mechanical properties can also affect cancer cell phenotypes as cells can sense the physical status and array of peri-tumoral ECM and respond by adapting their phenotypes to the new environment. For instance, an increase of ECM stiffness induces cancer cells to gain an elongated shape and re-enforce focal adhesions, cytoplasmic protrusions, and ability to exert high-traction forces (Peyton et al. 2008; Koch et al. 2012; Ringer et al. 2017). An increased collagen stiffness also

affects cells in microenvironment surrounding tumor by modulating remodeling cytoskeleton of fibroblasts and cancer associated fibroblasts (CAFs) (Stylianou et al. 2019).

Particular attention should be given to the different invasion modes and cancer cells ability in switching them or "plasticity." The different invasion modes are related to different cell phenotypes and cell shape seems to predict ECM-driven 3D cell invasion in breast cancer (Baskaran et al. 2020). Both individual and collective invasions can occur: the first mode concerns cells loosing cell–cell contacts, whereas the second one involves cells which still maintain cell–cell junctions. Cells which invade in a collective mode can move as multicellular streams, budding, or larger clusters, whereas cells invading individually can develop elongated-mesenchymal phenotypes, contractility-driven rounded-amoeboid, and filopodial spike-mediated invading strategies. Cells adopting an elongated phenotype are characterized by actin-rich protrusions, focal adhesion formation, MMPs activity, and actomyosin contractility at the rear of the cells (Pandya et al. 2017). In general, these mesenchymal-like cells develop a highly motile phenotype and can easily cross basement membranes, interstitial spaces, and endothelial barriers in a single-cell migration. The strong cohesive intercellular forces favor the collective invasion and offer potential advantages for tumor survival such as covering different cell roles and improving resistance to immunologic attack (Christiansen and Rajasekaran 2006). Transition from collective to single-cell invasion or tumor plasticity may enhance a metastatic ability (Friedl et al. 2012). Anyway, all migration movement or invasion mode is related to intracellular actin polymerization with development of different cytoskeletons and new cell phenotypes.

To colonize distant organs, cancer cells must start a journey through different body tissues, at first by spreading from the body epithelial outer layer to the neighboring deeper tissues and then by invading blood and lymphatic vessels. In the first step of their journey, cancer cells have to cross ECM of connective tissues which contains different biological barriers opposing cell migration. ECM "biological barriers" can physically opposite but also favor migration/invasion of cancer cells: the first barrier comprises the *basement membrane* (BM) which serves as a scaffold for all epithelial cells; under the BM, the second one is represented by an *interstitial loose connective tissue* called papillary and subpapillary dermis in skin, lamina propria in hollow organs, or interstitial stroma in solid organs; a third biological barrier may be constituted by the *deeper dense connective tissue* which contains higher amount of collagen fibers of different size and array. As an example, in skin under the subpapillary dermis, we find the reticular dermis layer whose collagen accounts for about 70% of the weight of dried tissue. Both density and collagen array change from the papillary and subpapillary dermis, where collagen fibers composed of thin fibrils are sparse and show a low aggregation, to the deeper reticular dermis which contains thicker collagen fiber bundles with a horizontal laminar organization, densely distributed and including long and thick fibrils (Ribeiro et al. 2013; Breitkreutz et al. 2013; Limbert 2017; Ueda et al. 2019). As shown in Fig. 10.1a, b ECM just beneath the epithelium includes thin loose collagen

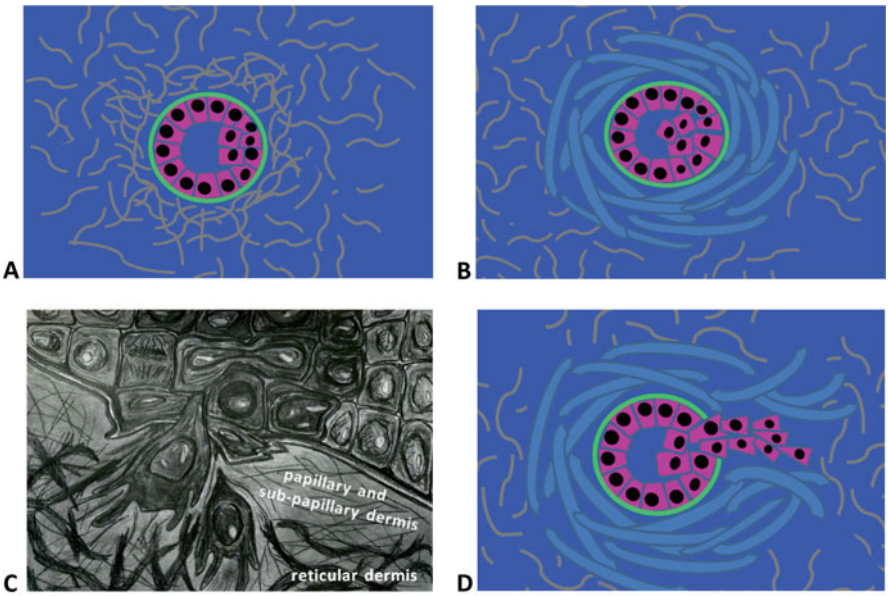

Fig. 10.1 Tumor-Associated Collagen Signatures (TACS). (**a**) In the first stage, a peri-tumoral deposition of collagen occurs (TACS-I). (**b**) When tumor grows, the collagen fibers around the pathological mass change their array and appear aligned and parallel to the tumor surface (TACS-II). (**c**) When cancer cells lose contact with each other and become able to penetrate the basement membrane, which is the first biological physical barrier opposing invasion, they can invade ECM of connective tissues. As an example, in skin cancer, cells pierce the basement membrane and then begin to migrate into the papillary and sub-papillary dermis containing sparse and thin collagen fibers. They further penetrate the depth of the tissue by invading the reticular dermis which contains thick collagen fibers. (**d**) With further development of the tumor mass, the collagen fibers develop a radial array that can favor cancer cell invasion (TACS-III)

fibrils, whereas the deep connective tissue layer comprises thicker collagen fibril bundles (Ottani et al. 1998).

The previously described biological barriers: (a) the BM, (b) the interstitial loose connective tissue/lamina propria/interstitial stroma, and (c) the deeper dense connective tissue, not only oppose cancer cells invasion but also offer anchorage and adhesion to MDA-MB-231 breast cancer cells which can better invade ECM by improving cell contraction and adapting their cytoskeleton (Byrne et al. 2021). In particular, type I and type III collagen assembled in fibrils and fibrillar fibronectin represent the main molecules composing physical scaffolds opposing and supporting the cells migratory movement. During tumor growth, changes of architecture of peri-tumoral collagen network are related to tumor progression and clinical prognosis (Egeblad et al. 2010; Conklin et al. 2011).

Markers of mammary carcinoma progression have been identified in a clinical-histological Tumor-Associated-Collagen-Signatures (TACS) classification. In a first step, only an increase in collagen deposition or desmoplasia around the tumor occurs

(TACS-I) and no metastasis are reported. When tumor mass grows in size, the randomly arranged peri-tumoral collagen fibers are substituted by straight and aligned collagen fibers showing a circumferential orientation (TACS-II), and at this histological stage, no clinical metastasis occurs. However, a further remodeling of the collagen fibers can occur, collagen fibers arrange running perpendicularly to the tumor boundary (TACS-III). Unfortunately, this radial collagen fiber array seems to be strongly related to a higher risk of cancer cell invasion into peri-tumoral microenvironment, because the stretched and radially arranged collagen fibers allow but also favor interfiber cancer cell migration and colonization in distant organs (Provenzano et al. 2008; Riching et al. 2014; Bredfeldt et al. 2014; Wang et al. 2018; Franchi et al. 2019). From a mechanical point of view, the remodeling of ECM promotes matrix alignment and compaction that favors higher tractions exerted by cancer cells (Anguiano et al. 2020) (Fig. 10.1a–c).

Besides collagen, fibronectin (FN) is one of the most abundant proteins mediating cell adhesion and migration in ECM. It can develop into fibrils that widely differ in their thickness, between 10 and 1000 nm (Kular et al. 2014). FN regulates collagen assembly, and the presence of collagen fibrils induces the formation of highly colocalized FN fibrils (Paten et al. 2019). In tumor stroma, both type I collagen and fibronectin are strongly increased and create a denser and mechanically stiffer tissue if compared to normal tissue (Tlsty and Coussens 2006; Rick et al. 2019). FN, arranged into a mesh of fibrils like collagen, but also connected to and within the (BM) of ECM, has been investigated as a possible ECM component which can favor cancer invasion and metastasis and is linked to cell surface receptors (integrins) (Kular et al. 2014). Tumor growth promotes compressive forces within tumor which are accomplished by a coordinated migration of invasive phenotypes in mammary carcinoma cells accompanied by fibronectin deposition and stronger cell–matrix adhesion (Tse et al. 2012).

Experimental mechanical stimulation applied to a collagen–fibronectin matrix highly enhanced invasion ability of HT1080 fibrosarcoma cancer cells only when FN was present. This suggests a primary potential role of this ECM component in governing the mechanical response of tumor cells and tumor progression (Menon and Beningo 2011). Even though its role in tumorigenesis and malignant progression has been highly controversial (Lin et al. 2019), it was reported that cancer-associated fibroblasts (CAFs), through increased contractility and traction forces, can assemble aligned FN matrix which seem to regulate directional migration of cancer cells (Erdogan et al. 2017). The stretching of fibronectin can activate a mechanical response pathway also in normal fibroblasts (Friedland et al. 2009), even though induced mechanical extension of single FN fibers seems to limit the spreading and migration of endothelial cells (Hubbard et al. 2015).

In this chapter, we present and critically discuss the changes of phenotypes and behavior of different breast cancer cell lines (MCF-7, MDA-MB-231) and colon cancer cells (LoVo-R) in 3D cultures mimicking the natural ECM barriers to cancer invasion. To mimic the natural BM, we prepared a layer of Matrigel at standard concentration (0.18 μg/mL) for PCR analysis (Albini et al. 1987), and at higher concentration (3.0 μg/mL) for scanning electron microscope (SEM) observations, as

at lower concentration, we never observed a continuous layer of this coating at SEM (Franchi et al. 2019). To reproduce the loose collagen meshwork below the BM (subpapillary and papillary dermis in skin, lamina propria in hollow organs or interstitial stroma in solid organs), we prepared low concentrated type I collagen network (50 μg/mL), whereas to mimic the deeper dense connective tissue we prepared high concentrated (3000 μg/mL) similar collagen on Millipore filters. We also tested FN (130 μg/mL) which functioning as ECM "biological glue" is critical for the attachment and guides directional movement of breast cancer cells (Yue 2014; Oudin et al. 2016).

Finally, just to mimic ECM collagen array of peri-tumoral denser connective tissues as described in TACS-II (collagen fibers arrayed parallel to the tumor boundary, i.e., orthogonal to the cancer cell invasion), we cultivated breast cancer cells on commercial lyophilized and compressed collagen membranes of tightly packed type I collagen fibers (Franchi et al. 2019).

10.2 2D Polystyrene Flask Cultures Partially Transformed in 3D Cultures Induce EMT in MCF-7 Breast Cancer Cells

The role that the physical aspect of substrate surface plays in governing the cancer cell phenotypes was evident when we casually observed an occasional line corresponding to a physical defect of some polystyrene flasks surface. An irregular culture surface may represent by itself a 3D substrate for both normal and cancer cells which become able to move in all spatial directions. When we use flat and smooth polystyrene flasks to cultivate cells, we perform 2D cultures. Where the flask surface was regular and very smooth, the low aggressive MCF-7 breast cancer cells grew exhibiting an epithelial shape and characteristics: cobblestone or flattened polygonal cells showed cell–cell contacts by short filopodia and some of them looked melted together by intercellular tight junctions. Only few globular isolated cells rich in cytoplasmic microvesiscles were detectable (Fig. 10.2a–d). But when the flask surface where cells were seeded showed a line, about 10 μm deep, the 2D substrate transformed the 2D cultures to 3D cultures and the cells which were very next to the flask line completely changed their shape exhibiting a morphological aggressive phenotype: these cells assuming an elongated or fusiform shape developed a morphological epithelial–mesenchymal transition (EMT). Moreover, some of them partially lost intercellular adhesiveness and seemed also to migrate and even cross the line. We demonstrated that changes of the physical aspect of the flask surface, such as a line, seem to induce migration in the low aggressive MCF-7 cells (Fig. 10.2a–d). They seem to adapt to the new microenvironment: to cross the line the cells develop a long filopodia, which exhibiting lateral short filopodia runs from one edge of the line to the other one (Fig. 10.2b) or a lamellipodia (Fig. 10.2d).

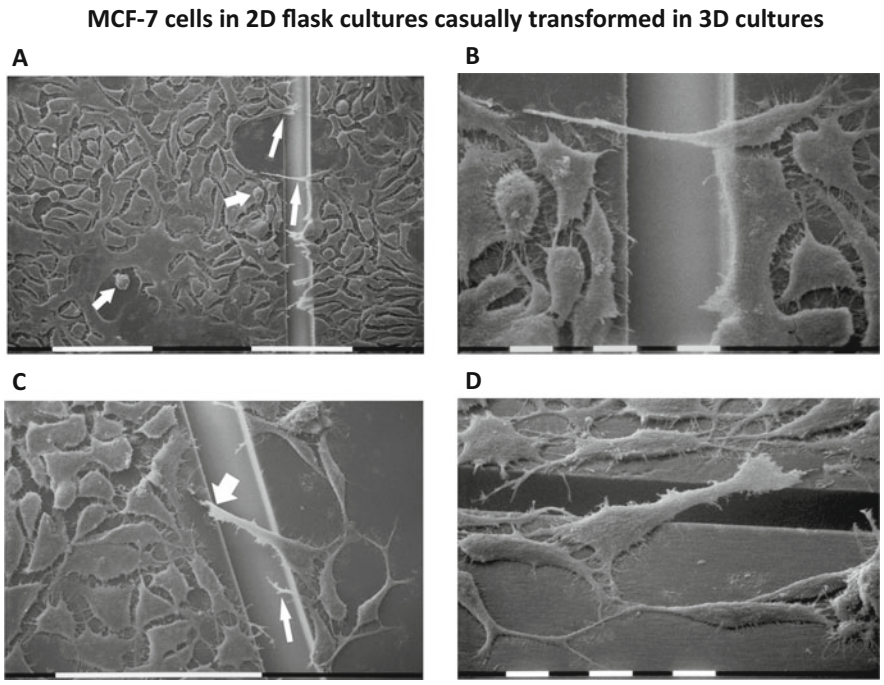

Fig. 10.2 MCF-7 cells on polystyrene flask 2D culture observed at SEM. (**a**) MCF-7 cells appear like flattened and smooth epithelial polygonal cells in contact with each other by short filopodia or in tight contact so to appear melted. Very few globular isolated cells with cytoplasmic microvesicles are detectable (large arrow). On the right of the picture, an occasional and regular line of the flask surface is present. MCF-7 cells very next to the line assume a mesenchymal elongate shape (thin arrows) to cross from one side to the other of the line edges. Note that cells crossing the line do not adhere to the bottom of the line (White bar = 100 μm). (**b**) A higher magnification of the previous picture shows a crossing cell developing a long filopodia. The surrounding cells appear like epithelial poligonal flattened cells (White bar = 10 μm). (**c**) The MCF-7 cells next to the flask line partially lose their cell–cell contact, exhibit an elongated and fusiform shape and develop lamellipodia (large arrow) or long filopodia (thin arrow) which demonstrate that they are moving. All the other cells show an epithelial phenotype and look in contact with each other (White bar = 100 μm). (**d**) A higher magnification of the previous picture shows elongated cells crossing the line and developing filopodia or lamellipodia (White bar = 10 μm)

Cancer invasion consists of a translocation of the cancer cell body and must be considered as a heterogeneous and adaptive process of cancer cells involving morphological changes and generation of cell polarity (Pandya et al. 2017). Physical aspect (line) of a culture substrate can affect phenotypes of low-aggressive breast cancer MCF-7 cells which develop more aggressive morphological shapes. In particular, we observed that these cancer cells cultivated on flasks in 2D cultures appeared isolated and developed EMT phenotypes when they casually grew on an irregular line which transformed the 2D culture in a 3D one (Balachander et al. 2015).

10.3 MCF-7 Breast Cancer Cells in 2D and 3D Cultures (Millipore Filter, Concentrated Matrigel-Covered Millipore, Low Concentrated Type I Collagen-Covered Millipore and Membrane of Densely Packed Collagen Fibers)

To investigate if ECM can affect phenotypes of a low aggressive breast cancer cell line (MCF-7), we reproduced in vitro the biological barriers which cancer cells must invade in order to colonize distant organs. The first biological barrier of outer ECM which cancer cells have to breach is the BM. To obtain continuous layer of culture substrate mimicking the BM and visible at SEM, we covered a Millipore filter with a thick layer of Matrigel. Then we also investigated how others deeper matrix biological barriers, which were mimicked with different concentrations and array of type I collagen fibrils or using a membrane of densely collagen fibers, could induce changes of MCF-7 cancer cell phenotypes.

The BM is a specialized form of ECM which underlies or surrounds most tissues, including epithelial, endothelial, muscle, and adipose tissues. BM is a sheet-like structure of more than 200 nm thickness composed by two independent polymeric networks, one of laminin and one of type IV collagen, which are probably linked by several additional ECM proteins, including nidogen and perlecan (Breitkreutz et al. 2013; Jayadev and Sherwood 2017; Scott et al. 2019). Glycoproteins and PGs, including fibulin, hemicentin, SPARC, agrin, and type XVIII collagen are also present in BMs, creating biochemically and biophysically distinct structures serving different functions (Jayadev and Sherwood 2017). BM was described at the transmission electron microscope (TEM) as composed of two layers: the lighter-stained layer closer to the epithelium and called lamina lucida, and the denser-stained layer one closer to the connective tissue and called lamina densa.

BM physically supports epithelia but also acts like a tissue scaffold separating but also connecting two different tissues: BM laminin interacts with both integrins on epithelial cell surfaces and type VII collagen of stromal anchoring fibrils which interact with types I and III collagen fibrils of ECM. At the same time, also BM provides essential functions of barrier as it separates epithelial cells from stromal connective tissues: usually cells cannot pass through the very thin pores (10–140 nm) of this barrier without degrading it. BM is an important regulator of cell behavior because it sends signals to epithelial cells about the external microenvironment. Moreover, BM is also a component of blood vessels and represents an extracellular microenvironment sensor for endothelial cells in intravasation events of cancer cells (Kalluri 2003a, b). To mimic the natural BM, we prepared a highly concentrated (3.0 μg/mL) layer of Matrigel because at standard concentration (0.18 μg/mL), we never observed at SEM the physical presence of this coating. Matrigel mimics the because it contains laminin, entactin/nidogen, type IV collagen, and heparan sulfate proteoglycans. Moreover, we prepared a layer of low concentrated type I collagen (50 μg/mL) as coating for a Millipore filter so to reproduce the

collagen array in ECM of loose connective tissues (subpapillary and papillary dermis, lamina propria, interstitial stroma).

Both 2D and all 3D cultures were observed at the SEM to evaluate the changes of cancer cells phenotypes growing on ECM components. Always function is related to shape but at the same time structural morphology depends on function. The morphological investigations on the shape of cancer cells allow us to better understand their behavior during invasion of the surrounding tissues and the biological events which lead to metastasis.

MCF-7 cells cultivated on smooth polystyrene substrate displayed epithelial phenotypes: cobblestone flattened polygonal shape with cell–cell contacts through many thin and short filopodia. No microvesicles were detectable on their cytoplasmic surface (Fig. 10.3a).

When the same MCF-7 cells were cultivated in a 3D culture on a Millipore filter, they lost their flattened epithelial phenotype observed in 2D flask cultures. All cells still appeared gathered but exhibited a globular shape and developed a collective invasion ability through an ameboid movement when they crossed the Millipore holes (Fig. 10.3b).

This invasion mode was not directly observed when the same cells were seeded on a Millipore filter covered with concentrated Matrigel (3.0 μg/mL), which appeared like a relative thick and very smooth layer: MCF-7 cells still maintained strong cell–cell contacts but tried to invaginate the thick Matrigel layer and developed short invadopodia breaching the artificial basement membrane (Fig. 10.3c). It was reported that laminin receptors and collagen IV of BM can induce EMT thus favoring cancer progression (Scott et al. 2019).

When MCF-7 cells were cultivated on a Millipore filter covered by low concentrated (50 μg/mL) type I collagen, mimicking ECM of the loose interstitial connective tissues below the BM, the fibrils occluded all the Millipore filter pores thus partially limiting the invasion through an ameboid movement. MCF-7 cells completely changed their phenotypes as many of them looked like isolated cells exhibiting a mesenchymal elongated shape which suggested an EMT induced by collagen fibrils. Cells showed also large lamellipodia strongly adhering to collagen fibrils and binding to them, but other ones developed intercellular cytoplasmic connections morphologically corresponding to tunneling nanotubes (Fig. 10.3d).

If MCF-7 cells are cultivated on a membrane of densely packed fibers of type I collagen mimicking TACS-II, the stage of breast cancer with collagen fibers densely packed and parallel to the tumor boundary and no clinical metastasis, they show grouped globular and few polygonal-shaped cells with cell–cell contacts and short microvilli on their surface, but they seem unable to invade the collagen membrane (Fig. 10.3e, f) (Provenzano et al. 2008; Franchi et al. 2019).

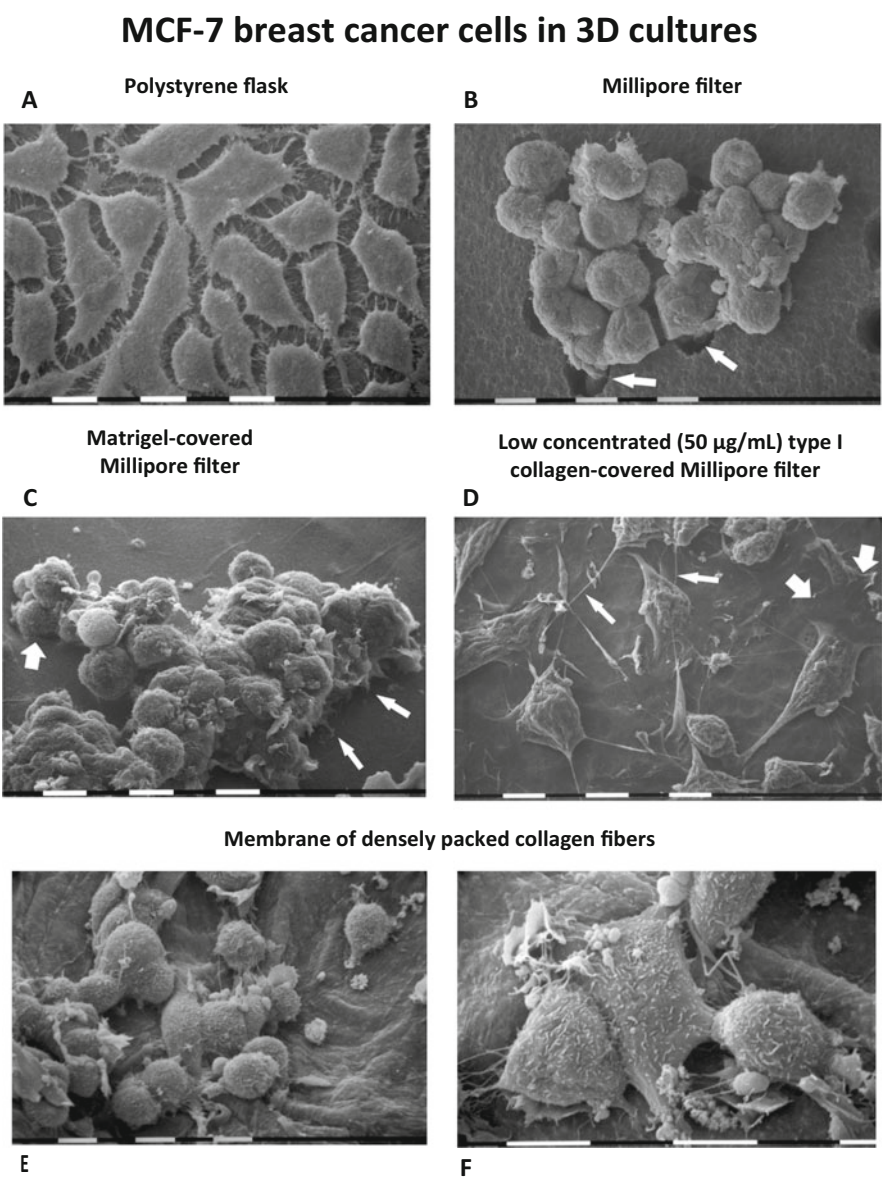

Fig. 10.3 SEM analysis of MCF-7 cells. (**a**) MCF-7 cells cultivated on polystyrene flasks in 2D cultures show epithelial phenotypes with cell–cell contact through many thin and short filopodia. (**b**) MCF-7 cells on the Millipore filter in 3D cultures show a globular shape and are grouped together. They can easily pass through the free Millipore pores (arrows) (White bar = 10 µm). (**c**) MCF-7 cells on Millipore filter covered by a concentrated Matrigel (3.0 µg/mL) in 3D cultures. Concentrated Matrigel appears like a relatively thick and very smooth layer on which very grouped globular MCF-7 cells array in tight contact with each other. They intimately adhere to Matrigel and it seems that they are invaginating the collagen fibrils (large arrow). They develop cytoplasmic protrusions which might morphologically correspond to invadopodia (thin arrows) (White bar = 10 µm). (**d**) The same MCF-7 cells cultivated on a Millipore filter covered by low

10.4 MDA-MB-231 Breast Cancer Cells in 2D and 3D Cultures (Millipore Filter, Concentrated Matrigel-Covered Millipore, FN, Low and High Concentrated Type I Collagen-Covered Millipore and Membrane of Densely Packed Collagen Fibers)

To explore whether ECM can affect phenotypes of a high aggressive breast cancer cell line (MDA-MB-231 cells), we reproduced in vitro the biological barriers which cancer cells have to break and invade in vivo to develop metastasis. We reproduced the first biological barrier of outer ECM, the BM, by covering a Millipore filter with a thick layer of Matrigel, but to mimic the other deeper matrix biological barriers, we covered a Millipore filter with different concentrations and array of type I collagen fibrils or we used a collagen membrane of densely packed collagen fibers.

Both 2D and 3D cultures were then observed at SEM to detect eventual changes of cancer cells phenotypes and cytoplasmic protrusions when cells grow on different ECM components. When MDA-MB-231 cells are cultivated on smooth polystyrene flasks in 2D cultures, they show different isolated phenotypes exhibiting cytoplasmic microvesicles: elongated fusiform-shaped cells, globular ones, and few flattened polygonal ones (Fig. 10.4a). When the same cells are cultivated on a Millipore filter in 3D cultures, they do not change their phenotypes but exhibit more microvesicles on their cytoplasmic surface. Single isolated cells can migrate through the Millipore holes developing filopodia or lamellipodia (Fig. 10.4b). It is interesting to observe that MDA-MB-231 cell phenotypes do not drastically change from 2D to 3D cultures. The thick layer of Matrigel covering the Millipore filter and mimicking the BM does not induce any phenotypes changes in MDA-MB-231 cells even though cells seem to better adhere to the substrate and some of them develop ventral cytoplasmic protrusions which are morphologically comparable to invadopodia (Fig. 10.4c). This data confirms the results of Anguiano et al. (2017, 2020) who demonstrated that at high concentrations, Matrigel slows down cancer cells migration, possibly due to excessive attachment, whereas at low concentration, it may facilitates migration. From a morphological point of view, at the standard concentration, Matrigel distribution does not covers all the Millipore pores so indirectly allows free cancer cell migration.

Fig. 10.3 (continued) concentrated (50 μg/mL) type I collagen fibrils occluding all the Millipore filter pores in a 3D culture. More flattened and isolated cells show a mesenchymal elongated shape with large lamellipodia binding collagen fibrils (large arrows), and intercellular cytoplasmic connections which morphologically correspond to tunneling nanotubes (thin arrows) (White bar = 10 μm). (**e**) MCF-7 cells cultured on a commercial membrane of densely packed collagen fibers appear grouped with cell–cell contacts and show a globular shape, but develop many microvilli on their surface (White bar = 10 μm). (**f**) At higher magnification, a polygonal and globular cell adhering to the membrane of densely collagen fibers are in tight contact and develop many cytoplasmic microvilli (White bar = 10 μm)

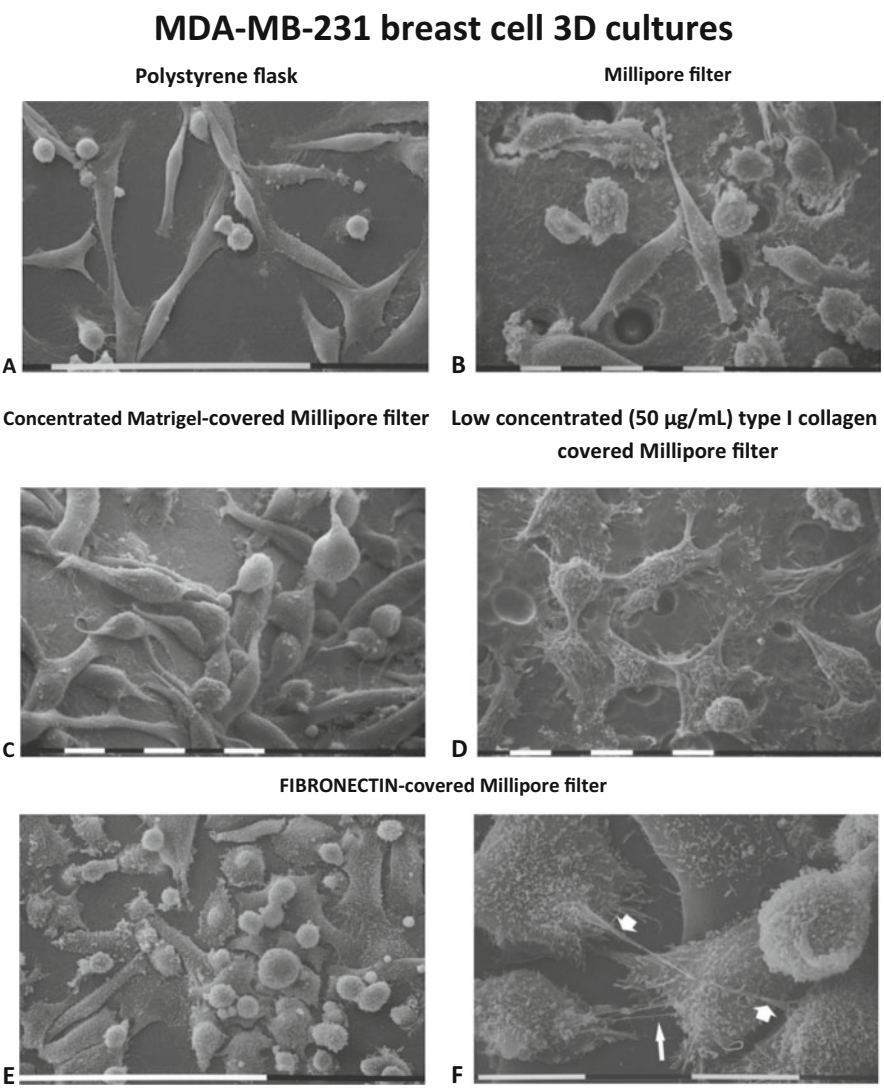

Fig. 10.4 SEM analysis of MDA-MB-231 cells. (**a**) MDA-MB-231 cells cultivated on polystyrene flasks in 2D cultures display different isolated phenotypes exhibiting cytoplasmic microvesicles: elongated-fusiform shaped cells, globular ones, and flattened polygonal ones (White bar = 100 µm). *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**b**) MDA-MB-231 cells on Millipore filter in 3D cultures show the same phenotype described in flask cultures but microvesicles are more present. Individual cells are migrating through the free Millipore filter holes (White bar = 10 µm). (**c**) MDA-MB-231 cells in 3D cultures on concentrated Matrigel-covered Millipore. The three phenotypes observed in Millipore filter cultures are still visible: many isolated elongated-fusiform and globular cells and fewer flattened polygonal ones (White bar = 100 µm). (**d**) MDA-MB-231 cells in 3D cultures on low concentrated (50 µg/mL) type I collagen-covered Millipore. The three isolated previously described different phenotypes of MDA-MB-231 cells are still recognizable but show more microvesicles on their cytoplasmic surface. *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological

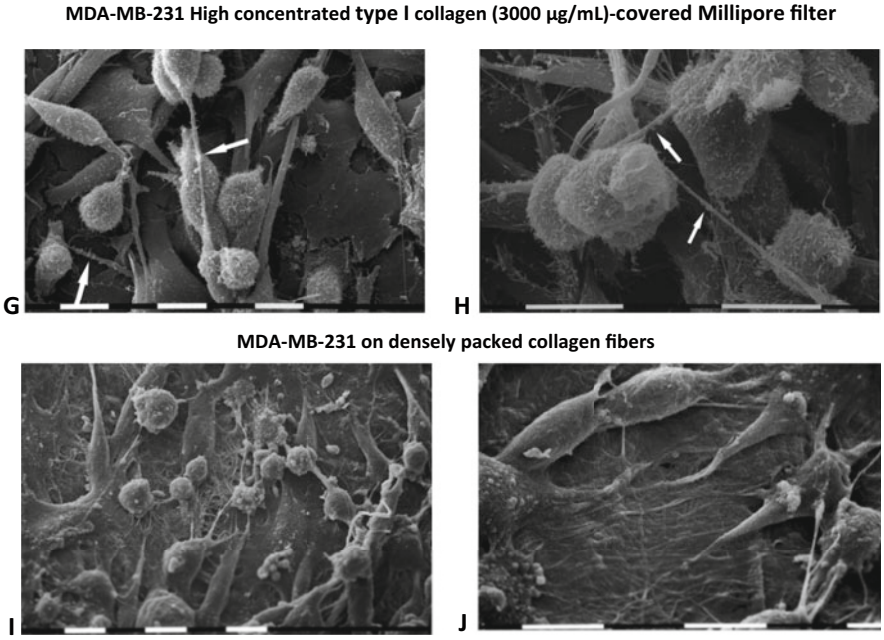

Fig. 10.4 (continued) Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**e**) MDA-MB-231 cells in 3D cultures on Millipore filter covered by fibronectin (130 µg/mL) occluding all filter holes. MDA-MB-231 cells cultured on fibronectin seem to be more grouped and better adhere to the substrate. They show many "cobblestone" flattened polygonal cells and globular-shaped ones, but only a few elongated ones. All cells exhibit many short filopodia or microvilli and microvesicles (White bar = 100 µm). *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**f**) MDA-MB-231 cells in 3D cultures on Millipore filter covered by fibronectin (130 µg/mL). Polygonal cells show microvilli and appear to be connected by thin single TNTs (thin arrow) and forming thicker ones composed of single thin TNTs tightly bundled together (large arrows). On the right, a globular cell exhibits many microvesicles on the cytoplasmic surface (White bar = 10 µm). *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**g, h**) MDA-MB-231 cells in 3D cultures on Millipore filter covered by high concentrated type I collagen (3000 µg/mL). Breast cancer cells grow in more than one layer, but no tight cell–cell contact is visible. Only the flattened polygonal cells lay in direct contact with the collagen fibrils, whereas both the elongated or fusiform cells and globular ones grow over the flattened ones. All cells show both microvilli and microvesicles. TNTs were apparently distinguishable in all phenotypes and long and thin filopodia originating from elongated cells exhibit exosomes and microvesicles (arrows) on their surface (White bar = 10 µm). *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**i**) The MDA-MB-231 cells attached to densely packed and parallel collagen fibers include a few isolated flattened polygonal cells, globular and elongated or also "squid"--shaped ones developing lamellipodia. All cells produce many microvesicles. Thin intercellular connections corresponding to TNTs are visible between adjacent cells. *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release. (**j**) In areas where fibrils of the collagen membrane are more exposed, elongated and fusiform shaped cells appear arrayed parallel to the fibrils axis (White bar = 10 µm). *From CELLS, 2020, 9, 2031,* Franchi M. et al. Extracellular Matrix-Mediated Breast Cancer Cells Morphological Alterations, Invasiveness, and Microvesicles/Exosomes Release

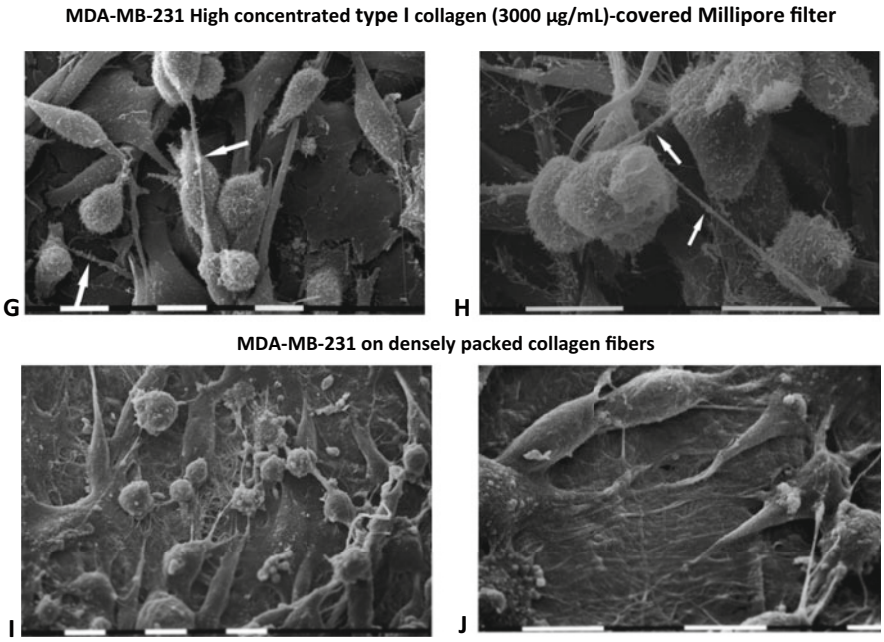

Fig. 10.4 (continued)

The MDA-MB-231 cells growing on low concentrated type I collagen (50 µg/mL) network still show the same phenotypes described in previous cultures, but cells display more evident and numerous cytoplasmic microvesicles (Fig. 10.4d). As both Matrigel and low concentrated collagen meshwork do not affect MDA-MB-231 phenotypes, we investigated in our experiments if FN, another ECM fibrillar component, could affect breast cancer phenotypes. Differently from previous samples, MDA-MB-231 cells cultured on fibronectin show many "cobblestone" flattened polygonal cells and globular shaped ones and only few elongated ones mainly growing on the previous ones. All cells appear more grouped with cell–cell contacts but exhibit many microvesicles and microvilli (Fig. 10.4e). Some of them are connected by thin single and thicker composed intercellular cytoplasmic protrusions which are morphologically comparable to tunneling nanotubes (TNTs) (Franchi et al. 2020a) (Fig. 10.4f). These data are explained considering that tested FN is arrayed in a network, so that the main effect of FN is just to favor cell adhesion. Only highly aligned FN is strongly related with EMT and high-speed migration (Wang et al. 2018). However, the presence of many microvesicles and the intercellular connections (TNTs) which allow a direct transfer of microvesicles and mitochondria between the connected cells suggest an increased metabolic activity in MDA-MB-231 cells. The observation of an increased number of polygonal cell phenotypes, which showing many microvesicles cannot be morphologically considered as low aggressive cells, suggest MDA-MB-231 cells develop a high adhesion to the FN substrate. Park and Helfman (2019) demonstrated that MDA-MB-231 cells develop

a high FN expression in 3D suspension culture and an increase of FN facilitates cell-attachment via integrin β-5. FN matrix can affect both cancer cells phenotypes and guide migration via integrins which connecting FN fibrils to cell's cytoskeleton might act as "stretch sensors" representing a "mechanical continuity" between the inner and outer environment of the cells (Kular et al. 2014; Oudin et al. 2016).

To observe possible stronger phenotypic changes, we covered the Millipore filter with higher concentrated type I collagen (3000 μg/mL). In these cultures, the previously described phenotypes showing both microvilli and microvesicles are equally distributed and grow in more than one layer: both elongated cells showing a fusiform shape and globular ones lay on flattened and polygonal ones which are the only ones in direct contact and adhering to the collagen fibrils. In elongated or fusiform cells, the higher concentration of type I collagen seems to induce the development of long and thin filopodia with exosomes and microvesicles on their surface. All phenotypes show microvilli and microvesicles (Fig. 10.4g, h). The higher concentration of collagen fibrils seems to further favor the metabolic activity and adhesion of MDA-MB-231 cells.

To mimic the collagen fiber array described in TACS-II (collagen fibers run parallel to the tumor boundary, i.e., orthogonal to the direction of cancer cell invasion) and corresponding to patient with no metastasis, we cultivated breast cancer cells on a commercial lyophilized and compressed membrane of tightly packed type I collagen fibers. It was described that at this stage, the remodeled collagen arrangement does not allow breast cancer cell invasion both in vivo and in vitro (Provenzano et al. 2008; Franchi et al. 2019). Cells include very few isolated flattened polygonal cells adhering to collagen fibrils of the membrane, globular and many elongated-fusiform ones and "squid"-shaped ones showing lamellipodia. All cells show microvesicles and TNTs (Fig. 10.4i). Where collagen fibrils are more exposed the fusiform cells are arrayed parallel to the fibril axis (Fig. 10.4j) (Franchi et al. 2020a).

10.5 Migration Test and Real-Time PCR of MCF-7 and MDA-MB-231 Cells

The migration/invasion process of cancer cells is the expression of their invasiveness properties. MDA-MB-231 cells have a higher ability in migrating/invading vs. MCF-7 cells, thus confirming the worst clinical prognosis of some patients. BM (Matrigel at standard concentration: 0.18 μg/mL) seems not to strongly affect both MCF-7 and MDA-MB-231 breast cancer cells phenotypes because when they are cultivated on both Millipore filter and Matrigel-covered Millipore filter all cells show the same rate of migration. However, when cells grow on collagen fibrils network-covered Millipore filter, MDA-MB-231 cells show a higher invasion capacity, whereas MCF-7 cells even decrease their ability in penetrating the barrier of collagen fibrils (Fig. 10.5). This different behavior between the different cancer

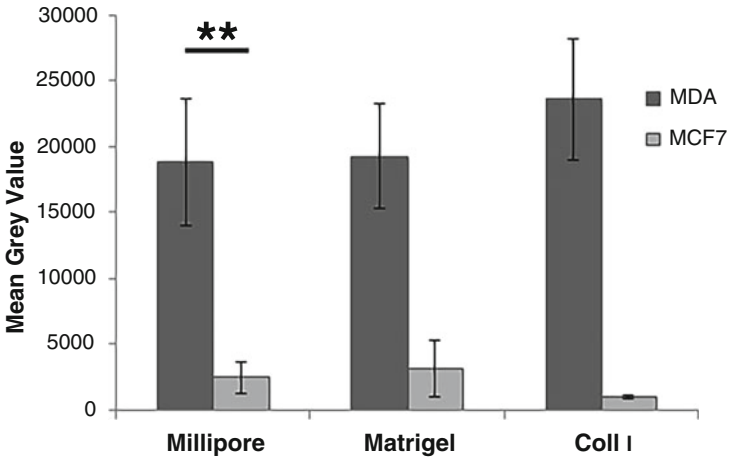

Fig. 10.5 Migration of breast cancer cells cultured on different substrates. MDA-MB-231 and MCF-7 cells were seeded on Millipore filters or Millipore filters covered with Matrigel (0.18 μg/mL) or collagen type I and incubated for 5 h. Double asterisks indicate statistically significant differences ($p \leq 0.01$). *From J. Clin. Med. 2019, 8, 213,* Franchi M. et al., *Collagen Fiber Array of Peritumoral Stroma Influences Epithelial-to-Mesenchymal Transition and Invasive Potential of Mammary Cancer Cells*

cell lines in contact with collagen might be explained by the strong and higher increase of MMP-2 and MMP-9 expression observed in MDA-MB-231 but not in MCF-7 (Fig. 10.6). It is well known that the release of MMPs from cancer cells cause an ECM digestion. This suggests an individual mesenchymal protease-dependent invasion mode for the MDA-MB-231 cultivated on a collagen network which instead represents a resistant biological barrier for the collective ameboid-dependent invasion of the MCF-7 cells (Bozzuto et al. 2015). In fact, if MCF-7 cells did not follow a protease-dependent invasion mode which creates wide spaces in ECM through MMPs of invadopodia, the very small pores size of the relative dense collagen network could not allow the crossing of cancer cell nuclei (Wolf et al. 2013). Anyway, collagen substrate seems to affect the MCF-7 cells phenotypes which show mesenchymal, elongated, or fusiform phenotypes (Fig. 10.3c): in fact, they display a VIM and FN-expression improvement and loss of cell–cell contacts as confirmed by a strong decrease of E-cadherin which was not observed in MDA-MB-231 cells collagen group (Khalil et al. 2017) (Fig. 10.6). It was reported that cadherin complexes act also as mechano-transducers that can sense changes in tension and trigger adaptive reinforcement of intercellular junctions (Leckband and de Rooij 2014).

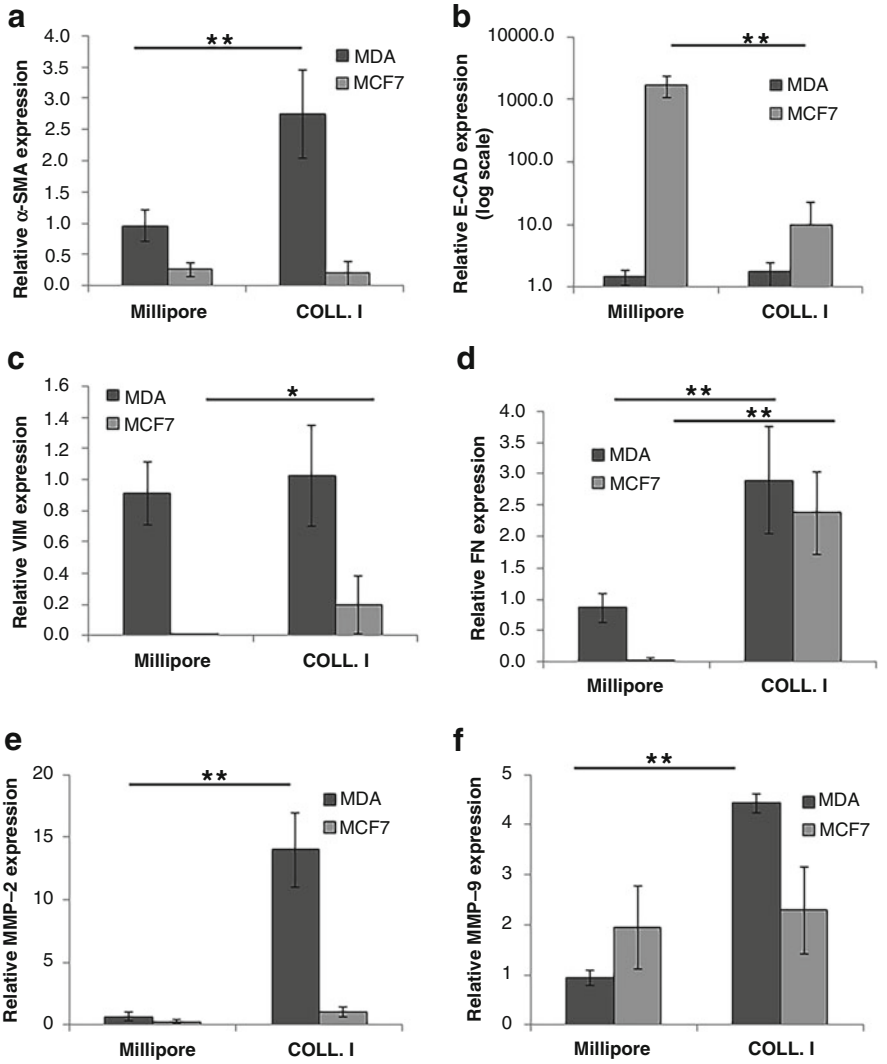

Fig. 10.6 Type I collagen induces striking changes in EMT markers and significant alterations in the expression of ECM components of breast cancer cells. Real-time PCR analysis of a-SMA, E-cadherin, vimentin, fibronectin, MMP-2 and MMP-9 on MDA-MB-231 and MCF-7 cells seeded on uncoated Millipore filter or filter covered with collagen type I and incubated for 24 h. Expression was normalized to GAPDH expression. Single and double asterisks indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively). *From J. Clin. Med. 2019, 8, 213,* Franchi M. et al., *Collagen Fiber Array of Peritumoral Stroma Influences Epithelial-to-Mesenchymal Transition and Invasive Potential of Mammary Cancer Cells*

10.6 Collagen as a Triggering Factor Inducing EMT in Breast Cancer Cells

The irregular surface of 3D cultures represents the first factor favoring the EMT of breast cancer cells, particularly evident in low aggressive (MCF-7) vs. higher aggressive (MDA-MB-231) cells, but also in 3D cultures, Matrigel (BM) does not affect so much the morphological phenotypes of all breast cancer cells. This data may be explained by the fact that even cancer cells like all normal epithelial cells lie, adhere, and grow in direct physical contact with a very flat and smooth scaffold, which epithelial cells sense and recognize as the BM (a smooth 2D surface). But when even some of the low aggressive MCF-7 cells lose their cell–cell contacts and breach, the BM they come in contact with a new unknown microenvironment represented by the ECM: very rough and porous collagen fibril loose meshwork, GAGs, PGs, glycoproteins, and water. They find a new and never sensed irregular and hostile physical microenvironment, so they adapt their phenotypes by developing cytoplasmic protrusions and phenotype changes which allow them to best invade ECM and reach vessels to provide nutrients. We can say that collagen itself induces morphological EMT in MCF-7 cells even though it could not enhance cell's ability to perform clinical metastasis, as it does not improve the production of MMP2 and MMP9 which allow cancer cells to penetrate lymphatic and blood vessels in metastatic process. This is confirmed by Invasion Test data which show that MCF-7 cells invasion decreases in collagen cultures, vs. Millipore and Matrigel substrates.

A molecular EMT in MCF-7 cultivated on type I collagen meshwork was, however, confirmed by a strong decrease of E-cadherin and an increase of both VIM and FN-expression. In conclusion, MCF-7, usually preferring a collective ameboid-dependent migration by squeezing through pliable barriers, could find a valid barrier in the irregular and resistant collagen network (Christiansen and Rajasekaran 2006; Bozzuto et al. 2015).

Deposition of collagen in human solid tumors is associated with higher incidence of metastasis (Ramaswamy et al. 2003). Even though collagen seems to not markedly promote morphological changes or EMT in MDA-MB-231 phenotypes, type I collagen fibrils even at low concentration are able to strongly improve MDA-MB-231 cells migration vs. the same cells cultivated in Millipore filter or in Matrigel-covered Millipore filter. Also, real-time PCR shows that type I collagen slightly promoted VIM-expression, but reveals a strong increase of α-SMA expression, FN-expression, MMP-2, and MMP-9 expression. The physical impact with the sub-BM collagen network could itself represent a "shock" for MDA-MB-231 cells which try to improve their invasiveness ability by increasing the production of MMPs. Moreover MDA-MB-231 cells showed at SEM a slight development of cytoplasmic microvesicles which contain also MMPs as reported in literature (Rilla et al. 2014).

Highly invasive MDA-MB-231 cells can induce metastasis in vivo so they are also capable of invading the deeper ECM including a higher concentration of collagen. In Millipore filter covered by high concentrated type I collagen

(3000 μg/mL) MDA-MB-231 flattened cells strongly adhere to the collagen network; on these cells, globular and elongated mesenchymal cells grow and develop microvilli, microvesicles, and TNTs which suggest an increased invasive potential (Franchi et al. 2020b). Also, when MDA-MB-231 cells were cultivated on FN-covered Millipore filter, they developed a higher number of flattened cells, expression of a good adhesion to FN, but showed a uniform and complete distribution of microvesicles on their cytoplasmic surface (Byrne et al. 2021). These different aspects in the same cells agree with the consideration that transition to an aggressive malignant phenotype is not an "all or nothing" event, so that we could not always observe morphological pure epithelial or pure mesenchymal phenotypes (Christiansen and Rajasekaran 2006).

Collagen and in particular the collagen fibrils/fibers array in tumor microenvironment plays a fundamental role in regulating tumor development and cancer cell invasion. Maller et al. (2013) found that collagen architecture in pregnancy can induce protection from breast cancer. They demonstrated that collagen organization, rather than density alone, is a fundamental contributor to induce an invasive phenotype. In fact, high density nonfibrillar type I collagen seems to suppress the tumor development by increasing junctional E-cadherin, upregulation of cell–cell junction genes, and downregulation of mesenchymal and metalloproteinase genes, whereas aligned fibrillar type I collagen favor cellular aggressive cancer cells phenotypes in mammary tumor cells and alters β1 integrin subcellular distribution.

Considering the particular peri-tumoral collagen array described in TACS-II and including the densely packed collagen fibers parallel to each other and to the tumor boundary, both MCF-7 and MDA-MB-231 cells seem to be unable to penetrate the collagen membrane used to mimic histological TACS-II stage. This ECM response is mediated by CAFs which seem to play an important role in changing the collagen array of tumor ECM: firstly opposing to cancer cell invasion (TACS-I and TACS-II) and then favoring the cancer cell invasion (TACS-III) (Provenzano et al. 2008; Egeblad et al. 2010; Conklin et al. 2011; Luhr et al. 2012; Bredfeldt et al. 2014; Costanza et al. 2017; Franchi et al. 2019, 2020a). Densely packed and parallel collagen fibers array observed in TACS-II are usually described in other anatomical situations and sites: the response of ECM in foreign body granuloma develops a tissue-encapsulation to limit diffusion of exogenous material by sheets of collagen fibers running parallel to the implant surface. Moreover, gingival ECM around titanium dental implants contains densely packed collagen fibers which following a circular array around the neck of the implants are presumably able to physically opposite virus and bacteria invasions from the oral plaque (Ruggeri et al. 1992, 1994). Which and how do the mechanical events which favor or also induce changes of collagen array in tumor ECM from TACS-II to TACS-III act? The densely packed, straight, and parallel collagen fibers array described in TACS-II are comparable to that of tendons. Tendon collagen fibers resist tension but when they are compressed, tenocytes change their phenotypes and ECM responds by developing a fibrocartilage, which protect both tenocytes and collagen fibers integrity. In particular, compressed tenocytes produce large amount of large PGs like aggrecan and versican which linking significant amount of water oppose compression (Docking

et al. 2013). However, considering the peri-tumoral microenvironment in cancer, HA acts as a modulator of the tumor microenvironment through signal transduction mechanisms, but also regulating the hydration and osmotic balance in tumor microenvironment. We also know that a very hydrated peri-tumor ECM could create interfibers spaces where cancer cells enter to invade the surrounding deeper tissue and a HA synthesis is increased in different cancers, such as breast, prostate, lung, colon, and ovarian (Tavianatou et al. 2019, 2021). Moreover, HA can increase the integrin-mediated transduction thus favoring cancer cell adhesion and invasion (Chopra et al. 2014).

If epithelial cell dissociation or loss of cell–cell adhesion related to E-cadherin decrease and changes of ECM physical array are the main factors which favor cancer cell invasion, clinical data suggest that cancer progression and metastasis occur also without E-cadherin deregulation (Khalil et al. 2017). Thus, the collagen fiber array in peri-tumor microenvironment seems to be the first actor in affecting tumor development and cancer cell invasion. The epithelial E-cadherin-positive MCF-7 cells, lacking the expression of membrane type 1-matrix metalloproteinase 1 (MT1-MMP)/MMP14) and not possessing the ability to proteolytically remodel the ECM, are able to invade collectively the ECM when they find a low dense collagen network rich in relatively wide spaces. Interestingly, repressing E-cadherin MCF-7 cells loose cell–cell contacts but do not invade high-density collagen which they can cross only when the MT1-MMP/MMP14 is ectopically expressed (Ilina et al. 2020).

10.7 LoVo-R Colon Cancer Cells in 2D Cultures and 3D Cultures (Matrigel-Covered Millipore, High Concentrated Type I Collagen-Covered Millipore)

The tumor microenvironment and in particular collagen array play an "epigenetic" role in influencing primary tumor growth, migration, and metastatic potential, but also can favor the ability of cancer cells to resist chemotherapy (Provenzano et al. 2008; Conklin et al. 2011; Franchi et al. 2019; Montagner and Dupont 2020). ECM concentration is an important and crucial parameter to evaluate drug screening. An increase of collagen concentration in 3D microenvironment induces an increase of the intracellular stiffness and motor activity of cancer cells and seems to influence the intracellular fluctuations even in the presence of chemotherapeutic and anti-MMP drugs (Kim et al. 2018). To evaluate the possible role that collagen can play in influencing the drug resistance in cancer, we cultivated a colon cancer cell line which is resistant to doxorubicin (LoVo-R) in a Matrigel-covered Millipore filter (control) and in a concentrated type I collagen network (3000 μg/mL). LoVo-R cells cultivated on Matrigel mimicking the basement membrane appeared grouped with relative cell–cell contacts through intercellular thin and short cytoplasmic processes; they exhibited a smooth surface and a polygonal and flattened shape which resemble

epithelial cells phenotype (Fig. 10.7a, b). The same cells cultivated on highly concentrated collagen showed a rounded shape and assembled in groups of few cells but some of them appeared isolated and showed a particular phenotype which resembles a "donut" shape (Fig. 10.7c, d). These cells displayed thin and short cytoplasmic protrusions which were in tight contact with the collagen fibrils or connected adjacent cells. We suggest that these cells were invading the collagen fibrils layer through a "funnel" invagination (Fig. 10.7e). From sectioned areas of the collagen-covered Millipore filter, we were able to detect thin and short cytoplasmic protrusions which developing from the ventral side of some cells penetrated the collagen fibrils network and morphologically corresponded to invadopodia (Fig. 10.7f). Moreover, a loss of collagen next to the cytoplasmic protrusions or invadodia suggests that a proteolitic activity next to the same protrusions occurred.

The ultrastructural data of LoVo-R cells cultivated in Matrigel or collagen substrates suggest that type I collagen induces EMT with drastic morphological changes of phenotypes observed in BM cultures: cells on collagen appeared more isolated and invading the collagen layer by developing invadopodia.

All these data suggest that collagen type I inducing an increase of cancer cells aggressiveness could also play a key role in favoring the development of a drug resistance. In fact, the previously described shock of MCF-7 and MDA-MB-231 cells when come in contact with the rough and irregular sub-BM microenvironment, could affect also LoVo-R cells, which developing a more aggressive phenotype could further increase their intrinsic resistance to therapeutic treatments. The development of drug resistance is a significant complication to successful of therapeutic procedures and seems partially favored by type I collagen composition of peri-tumoral ECM, which can significantly vary from patient to patient like clinical results suggest. The question is how can collagen improve cancer cells resistance to therapy? This could be partially explained because T-cell proliferation significantly reduces in a high-density matrix when compared to a low-density one (Kuczek et al. 2019). Other researchers suggest that tumor microenvironment could control the transport and thus efficacy of free doxorubicin in vitro (Brancato et al. 2018). Drug resistance is related to cancer cell lines but also tumor microenvironment: MDA-MB-231 cells displayed a stiffness-dependent response to the chemotherapeutic doxorubicin, whereas low aggressive MCF7 breast carcinoma cells cultured in the same conditions did not exhibit this stiffness-dependent resistance to the drug (Joyce et al. 2018). Anyway, the role of tumor ECM in modulating elasticity, adhesion, senescence, and EMT of MDA-MB-231 cells cancer cells seems associated with an improving of integrins upregulations (Poincloux et al. 2011; Mierke et al. 2011; Brandão-Costa et al. 2020; Byrne et al. 2021). Integrins are transmembrane proteins that physically connect cells to different proteins of ECM like fibronectin, vitronectin, collagens, and laminins but also to other cell surface receptors (Hynes 2002; Ringer et al. 2017). If the cadherin-based adherens junctions transduce the intercellular stresses (Leckband and de Rooij 2014), both chemical composition and all mechanical properties of the ECM are sensed by multimolecular complexes which are called focal adhesions (FAs) and include integrin receptors associated with the ECM (Geiger et al. 2009; Case and Waterman 2015). In

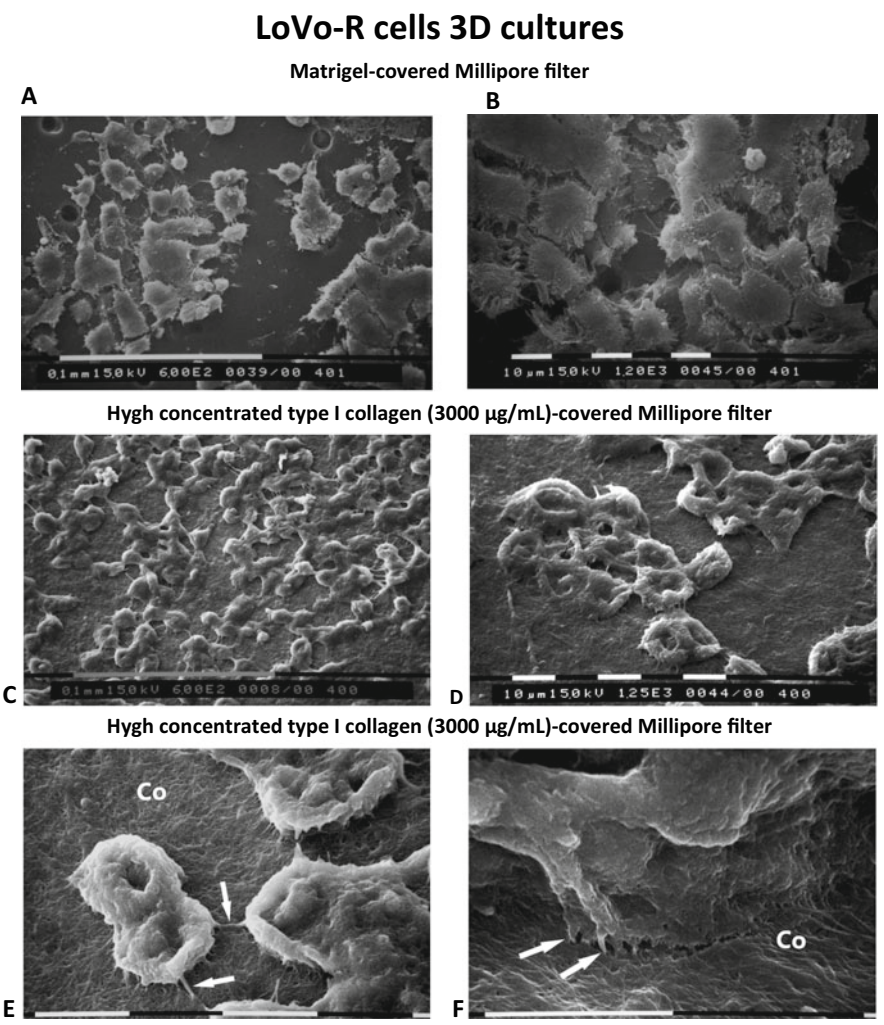

Fig. 10.7 SEM analysis of LoVo-R cells. (**a**) LoVo-R cells cultivated on a Matrigel-covered Millipore filter show a smooth surface and a polygonal-flattened shape like normal epithelial cells (White bar = 100 μm). (**b**) They appear distributed in a single cell layer and grouped with cell–cell contacts through intercellular thin and short cytoplasmic processes (White bar = 10 μm). (**c**) LoVo-R cells on high concentrated type I collagen (3000 μg/mL)-covered Millipore filter show rounded-shaped phenotypes assembled in small groups (White bar = 100 μm). (**d**) Some cells appear isolated and exhibit a particular phenotype that resembles a "donut" shape (White bar = 10 μm). (**e**) At a higher enlargement the "donut"-shaped cells show thin and short cytoplasmic protrusions in tight contact with the collagen fibrils or connecting adjacent cells (arrows). These cells are invading the collagen fibrils layer through a "funnel" invagination (Co = collagen) (White bar = 10 μm). (**f**) A lateral view of a sectioned collagen-covered Millipore filter shows that LoVo-R cells cultivated on high concentrated type I collagen (3000 μg/mL)-covered Millipore filter develop thin and short cytoplasmic protrusions from their ventral side which are penetrating the collagen fibrils network. These cytoplasmic protrusions correspond to invadopodia degrading adjacent collagen fibrils (arrows) (Co = collagen) (White bar = 10 μm)

particular, Ringer et al. (2017) described an outer FA layer including ECM-specific anchorage by integrin receptors, an intermediate layer where chemical and mechanical signals are processed, and an inner layer that is dominated by the actomyosin cytoskeleton of cells (Ringer et al. 2017). FAs complexes, linking integrins to the actin cytoskeleton either directly or indirectly by interacting with vinculin and α-actinin, act as mechanically elastic springs which enforce by talin unfolding when mechanical tension increases but remain small and short-lived when forces decrease in ECM. Therefore, they allow FAs to transmit the chemo-mechanical stimuli from ECM to the inner actomyosin cytoskeleton (Ringer et al. 2017). The main role of type I collagen in affecting the tumor progression is also supported by the fact that cells adhering to collagen type I by integrin α2β1 induce a very different signaling response compared to cells which bind collagen type IV with α1β1 integrin (Ivaska et al. 1999; Borza et al. 2012,; Ringer et al. 2017). To support the role for integrin expression and activation in regulating the development of oncogenic processes, it was reported that also hyaluronan, a soft polymeric glycosaminoglycan ECM component which is also considered a marker of breast cancer, can affect the cellular response by increasing the integrin mechano-transduction, thus suggesting the interaction of signaling between hyaluronan receptors and specific integrins. In particular, hyaluronan augments or overrides mechanical signaling by some classes of integrins to produce a spread morphology of cells otherwise observed only on very rigid substrates (Chopra et al. 2014). It is evident that in further experiments, it will be necessary to reproduce the in vivo conditions and include most of the ECM components in 3D cultures. Even though we explored the critical role of collagen and FN in affecting cancer cell phenotype and behavior vs. the BM, further investigations concerning the other molecules of ECM will help to better understand how tumor microenvironment could play a primary role in regulating cancer cell invasion. It is also plausible that only through evaluation and comparison of the interplay among all different ECM components, we will be able to suggest innovative therapeutic strategies to prevent or limit cancer invasion.

10.8 Conclusions: Take Home Message

1. Just a physically irregular surface of a culture substrate can transform 2D to 3D cultures simply because cells are free to move in all three spatial directions and may induce EMT in the low aggressive MCF-7 breast cancer cells.
2. In 3D cultures, a thick layer of Matrigel mimicking the BM does not promote changes in morphological phenotypes of both low aggressive MCF-7 and very aggressive MDA-MB-231 cells, probably because all epithelial cells recognize the smooth BM on which they usually grow.
3. In 3D cultures, a loose network of type I collagen fibrils mimicking ECM of sub-BM induces different effects on low aggressive MCF-7 cells vs. very aggressive MDA-MB-231 ones. Collagen favors the development of mesenchymal phenotypes morphology in MCF-7 cells but limits the invasion ability of the

same cells if compared to Matrigel cultures, as collagen induces a reduction in MMPs production in cells preferring an ameboid-invasion mode. On the contrary, type I collagen fibrils do not improve the EMT-related morphological changes in MDA-MB-231 cells which, however, in invasion test, show a strong increased ability to invade the collagen layer if compared to the same cells cultivated on Matrigel, as collagen seems to stimulate MMPs production.

4. In 3D cultures, nonaligned FN favors both MCF-7 and MDA-MB-231 cancer cells adhesion by developing more flattened and polygonal cells suggesting that nonaligned FN favors cancer cells adhesion. The increase of cytoplasmic microvesicles and the development of TNTs suggest an improve of cell–cell communications.
5. In 3D cultures, high concentrated type I collagen fibrils mimicking the deeper ECM induce breast cancer cells to grow in more than one layer, thus suggesting that concentrated collagen favors a further adhesion of cancer cells. The development of microvilli, microvesicles, long filopodia, and TNTs suggests that collagen may also stimulate communications among both adjacent and distant cells.
6. In 3D cultures, densely packed and parallel collagen fibers mimicking TACS-II stage in mammary tumor progression contrast both MCF-7 and MDA-MB-231 breast cancer cells invasion.
7. In 3D cultures of high concentrated type I collagen network, the LoVo-R colorectal cancer cells, resistant to doxorubicin, develop new morphological invaginating phenotypes and invadopodia. These morphological aspects suggest that the increased invasion ability of cancer cells could be related to the drug resistance.
8. Collagen, also in different arrays, could act as a physical factor inducing EMT in MCF-7 and MDA-MB-231 cells and drug resistance in LoVo-R cells.

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Chapter 11
Integrins in Cancer: Refocusing on the Tumor Microenvironment

Cédric Zeltz, Ning Lu, Ritva Heljasvaara, and Donald Gullberg

Abstract Despite having been characterized for several decades, the development of therapeutically useful compounds based on integrins has shown little success. In cancer biology, previous studies mainly focused on the roles of integrins in tumor cells and tumor vasculature, but currently, the role of integrins in cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) has entered the limelight. The TME is a complex meshwork of extracellular matrix (ECM) macromolecules filled with a collection of cells, including CAFs, blood vessel-associated endothelial cells, smooth muscle cells, pericytes, mesenchymal stem cells, and a variety of immune cells. As a main producer of ECM and paracrine signals, the CAF is a central cell type during ECM remodeling, but CAFs are also receiving increased attention as key players of immunosuppression and chemoresistance. Whereas the paracrine signaling by CAFs has been extensively studied in the context of tumor stroma interactions, the nature of the numerous integrin-mediated cell-ECM interactions occurring in the TME has remained understudied. We will in this chapter discuss integrins with a focus on the TME and the interplay between cancer cells, immune cells, and CAFs in the context of three solid tumor types. As an example of the multifaceted mode of action of the stromal integrin α11β1, we will summarize our current understanding on the role of this CAF-expressed collagen-binding integrin in these three tumor types and discuss data suggesting that blocking

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desmoplasia might have opposite effects in different tumor types. The recent discoveries of CAF heterogeneity and tumor specific effects of desmoplasia suggest that much remains to be learned about tumor stroma interactions. We suggest that integrin-based targeting of cancer cells and angiogenesis should be shifted to targeting of tumor-supportive CAF subpopulations, which so far has been a relatively unexplored venue.

11.1 Introduction

In solid tumors, the activated fibroblasts, or cancer-associated fibroblasts (CAFs), constitute a major population of cells in the tumor microenvironment (TME). CAFs have been documented to affect tumor growth, formation of stem cell niches, immunosuppression, metastasis, and chemoresistance (Multhaupt et al. 2016; Su et al. 2018). We recently summarized the role of CAFs and integrins in three desmoplastic tumor types (Zeltz et al. 2020). In this chapter, we will update this information, but with more of a focus on CAF-expressed integrins and extend our discussion to the role of TME-mediated fibrosis in tumor progression and chemoresistance (Fig. 11.1). We will stick to the three desmoplastic tumor types we previously discussed (pancreatic cancer, breast cancer, and lung cancer) and retain a certain focus on integrin α11 (Zeltz et al. 2019). We regard this as a timely subject since it is likely that in the coming years, there will be an increased interest in cellular TME interactions.

The *fibroblast* is a cell type of primary significance for extracellular matrix (ECM) production and remodeling in interstitial tissues (Nagalingam et al. 2018). Activated fibroblasts, i.e., *myofibroblasts* or *CAFs,* are crucial in the processes of wound healing and tissue- and tumor fibrosis, respectively. In accordance with this, experimental data show that fibroblasts use analogous "toolkits" to reorganize the ECM in these different conditions (Rybinski et al. 2014; Gullberg et al. 2016; Kalluri 2016). The key characteristics of normal tissue fibroblasts, myofibroblasts, and CAFs are summarized in information Boxes 11.1 and 11.2.

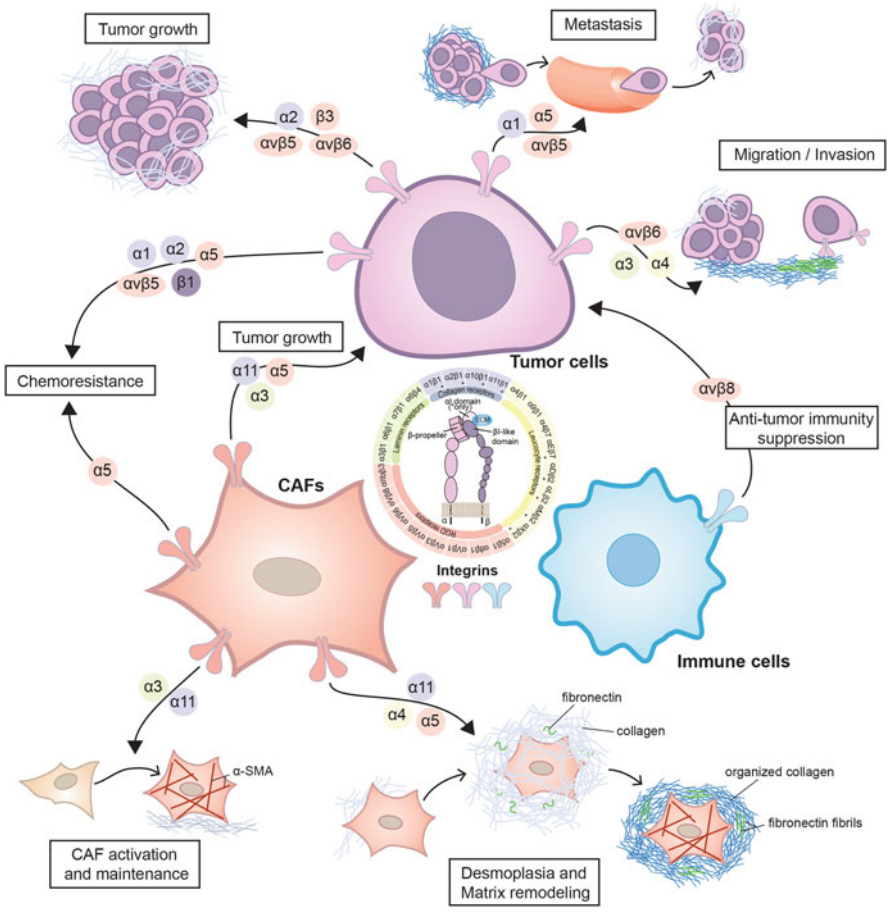

Fig. 11.1 Integrin functions in CAFs, tumor, and immune cells. The schematic summarizes integrin functions in pancreatic, lung, and breast cancer based on studies cited in Table 11.1. Integrins or integrin subunits involved in each process are indicated. Integrins are transmembrane receptors involved in extracellular matrix (ECM)–cell interactions. The integrin family is formed by 18 α and 8 β subunits that dimerize to form 24 integrins with distinct ligand specificities. Cancer-associated fibroblasts (CAFs) can differentiate into myofibroblastic CAFs (myCAFs), which show increased contractility due to alpha smooth muscle actin (α-SMA) expression. These cells, present in desmoplastic tumors, synthesize and remodel the ECM to increase stiffness, which in turn can affect tumor growth, invasion, and chemoresistance. A major CAF integrin involved in this process is α11β1. CAF integrins also act in a paracrine manner, via cytokine secretion, to promote tumorigenesis. Integrins expressed on tumor cells promote tumor growth, tumor invasion, and metastasis. Some tumor integrins confer chemoresistance and targeting αvβ5 integrin potentiates chemotherapy. Integrin αvβ8 on immune cells is responsible for antitumor immunity suppression

Box 11.1

The *fibroblast* is a cell type, of mesenchymal or neural-crest origin, that is nonepithelial, noninflammatory, and nonvascular. The fibroblast is a not only a key factor in processes involving synthesis of fibrillar collagens and other interstitial ECM components, but also an active cell type in integrin-mediated matrix remodeling as well as being a source of matrix metalloproteinases during tissue regeneration events (Multhaupt et al. 2016; Nagalingam et al. 2018). The transcriptional profile of fibroblasts varies with the anatomical location (Rinn et al. 2006). Cell lineage tracing has highlighted distinct origins of mouse fibroblasts in skin and cardiac tissue. Fibroblasts of mouse heart are derived from endocardium or epicardium (Moore-Morris et al. 2016). In mouse skin, a shared multipotent progenitor for papillary and reticular fibroblasts has been identified where subtypes of neonatal fibroblast are distinguished by a dynamic biomarker expression (Driskell et al. 2013; Guerrero-Juarez et al. 2019). Further heterogeneity of dermal fibroblasts arises from the complex cellular organization of hair follicles and due to the different embryonic origins of dermal fibroblasts (from dermomyotome in the posterior part of the body, from lateral plate mesoderm in the anterior part, and from the neural crest in the face). A dynamic expression of biomarkers also exists in human dermal fibroblasts with some notable differences compared to mouse skin (Philippeos et al. 2018). Recently, a comprehensive database was generated based on RNA-seq data from fibroblasts in mouse and human healthy and diseased tissues (fibroXplorer.com), and a summary of the data in this database was published (Buechler et al. 2021). The extensive analysis of the data generated a cross-tissue comparative atlas of fibroblasts gene expression and revealed a general organizing principle of the fibroblast lineage within and across organs. The study by Buechlet et al. describes two universal fibroblast lineages that express Dpt (dermatopontin) and Pi16 (peptidase inhibitor 16) or Dpt and Col15a1 (collagen XV) across tissues. The authors also demonstrate that these universal lineages give rise to eight types of tissue-specialized fibroblasts and in addition three types of activated fibroblast types during tissue injury.

Box 11.2

Myofibroblasts are activated fibroblasts considered to be contractile due to expression of the contractile isoform of actin, alpha smooth muscle actin (α-SMA) (Hinz et al. 2001; Tomasek et al. 2002). Myofibroblasts express αvβ1 integrin with a central role in transforming growth factor beta (TGF-β) activation in fibrotic conditions (Reed et al. 2015). Myofibroblasts were originally always thought to be depleted via apoptosis after completed wound healing (Tomasek et al. 2002; Schulz et al. 2018). However, mouse

(continued)

Box 11.2 (continued)
cardiac myofibroblasts have been observed to turn off α-SMA expression in the heart and form a cell type called matrifibrocyte with different properties than the undifferentiated premyofibroblasts (Fu et al. 2018). Emerging data thus suggest that myofibroblasts display more plasticity than previously thought. The finding that subsets of mouse skin myofibroblasts under certain conditions can differentiate into adipocytes further stresses the plasticity of myofibroblasts (Plikus et al. 2017).

Cancer-associated fibroblasts (*CAF*s) are fibroblast-like cells, of different origins, which are present in the TME. Sometimes used as abbreviation for carcinoma-associated fibroblasts, to specifically denote cells associated with epithelial-derived tumors. Demonstrated to be surprisingly heterogeneous. Several surprisingly heterogeneous CAF subtypes have been defined within tumor stroma. Pioneer work has defined three major types of fibroblasts in human and mouse pancreatic cancer ductal adenocarcinomas (PDACs): inflammatory CAFs (iCAFs), myofibroblastic CAFs (myCAFs) (Ohlund et al. 2017), and antigen presenting CAFs (apCAFs) (Elyada et al. 2019). Four major subclasses of CAFs in breast cancer (CAF-S1–S4), distinguished by different levels of α-SMA and fibroblasts activation protein (FAP) expression (Costa et al. 2018; Costa-Almeida et al. 2018) have also been described. Due to the plasticity and dynamic nature of the CAFs, it has been suggested that the CAF subtypes do not represent fixed cell types, but rather represent fibroblast "states" (Nurmik et al. 2019). Epigenetic changes do however result in more stable phenotypes (Marks et al. 2016; Eckert et al. 2019). Accumulating evidence indicates that some subpopulations of CAFs are tumor-supportive, whereas others are tumor-suppressive (Ireland and Mielgo 2018; Biffi and Tuveson 2018). In summary, CAFs have been demonstrated to act in a paracrine manner to affect different aspects of tumorigenesis, and via matrix synthesis and matrix remodeling to induce stiffness and hypoxia, which in turn also affect tumor growth. The comprehensive summary of fibroblast lineages described above also included data on transcriptional subtypes from human pancreatic ductal adenocarcinoma (PDAC) CAFs and nonsmall cell lung cancer (NSCLC) CAFs and demonstrated enrichment of an Lrrc15+ (leucine rich repeat containing 15) CAF subset (Buechler et al. 2021).

11.2 Tumor Fibrosis

Tumor desmoplasia is to a large part driven by paracrine and autocrine signaling of chemokines, cytokines, and growth factors. These factors regulate cell migration and cell proliferation, the secretion of ECM molecules, and the CAF-mediated crosslinking of fibrillar collagen matrices that often result in increased tissue stiffness and hypoxia (Kalluri 2016; Piersma et al. 2020; Zeltz et al. 2020). Additionally, ECM remodeling and reorganization are key factors that modify the properties of the

TME. The origin of CAFs varies with the tissue. Endogenous tissue fibroblasts, ADAM12^{+} perivascular cells and pericytes, have all been described as sources of CAFs (Dulauroy et al. 2012; Kalluri 2016; Ohlund et al. 2014). Moreover, through cell lineage tracing methods, a platelet-derived growth factor receptor alpha negative (PDGFRα^{-}) and clusterin^{+}- breast cancer CAF subset have been demonstrated to emerge from mesenchymal, nonhematopoietic bone marrow cells in the polyoma middle T (PyMT) oncogene-driven mouse mammary carcinoma model (Raz et al. 2018). The epithelial-to-mesenchymal transition (EMT) as a source of CAF seems to be restricted but contributes to form an invasive mesenchymal tumor cell phenotype and to create a niche for the emergence of cancer stem cells (Nieto et al. 2016). Furthermore, EMT indirectly affects the stroma. For example, Pastushenko et al. have convincingly demonstrated that the stroma evolves (composition, immune cells) as tumor cells progressed toward EMT in a spontaneous EMT genetic model (*Kras* $^{LSL\text{-}12GD}$*/p53* $^{fl/fl}$*/Lgr5CreER*) of squamous cell carcinoma (SCC) (Pastushenko et al. 2018). Westcott and colleagues have characterized breast cancer cell invasion in vitro and identified a switch of tumor cell state into a mesenchymal invasive state that is described as not being a completed EMT. The invasive tumor cells, named trailblazer cells, which initiate the path of invasive migration, were identified by a mesenchymal seven gene signature including *ITGA11* and *PDGFRA* (Westcott et al. 2015). Thus, when discussing CAF heterogeneity, it is essential to distinguish CAFs from: (1) EMT-derived cells that express varying mesenchymal biomarkers, (2) resident mesenchymal stem cells, and (3) trailblazer cells, which display a mesenchymal signature. So far, we are lacking biomarkers that can distinctly distinguish these different cell types.

11.2.1 CAFs in Desmoplastic TME

In the tumor stroma, cellular interactions of CAFs with the ECM contribute to CAF activation, tumor cell growth, tumor cell invasion, and metastasis, and also affect immunosuppression, chemoresistance, and the formation of stem cell niches. Some of these aspects have been discussed in previous reviews [see (Östman and Augsten 2009; Kwa et al. 2019; Chen and Song 2019; Zeltz et al. 2020)]. In this chapter, we will update the role of CAF interactions in pancreatic, mammary, and lung fibrotic tumors.

Since the role of integrins in the TME is understudied, we foresee new molecular data in years to come, which will include pairing of paracrine mechanisms with active integrins in different interacting cell types, including CAFs, as well as a deeper understanding of integrin-dependent chemoresistance mechanisms in response to tyrosine kinase inhibitors (Cruz da Silva et al. 2019).

The availability of integrin-binding sites in tightly packed collagen fibrils in vivo has been suggested to limit the activity of collagen-binding integrins (Zeltz et al. 2014; Woltersdorf et al. 2017). An emerging picture suggests that integrin-binding sites are to some extent made available during remodeling of the collagen fibril

surface thanks in part to proline-mediated flexibility (Chow et al. 2018; Zhu et al. 2018). However, this "microremodeling" is different from major matrix remodeling events including deposition of freshly synthesized collagens where the availability of integrin binding sites is not limited. In this context, CAFs in a growing tumor would be able to directly interact with the collagen matrix through collagen-binding integrins. In a fibronectin (FN)-rich matrix, a switch might occur to also include indirect linkages via α5β1 (Musiime et al. 2021).

Although several studies suggest that reorganization of collagen to a linearized and stiff matrix promotes tumor metastasis, the role of the ECM in tumorigenesis regarding its supportive or suppressive effects seems to vary with tumor type (Kai et al. 2019). A seminal study by Sahai and colleagues has shown that CAFs can pave the path for invading tumor cells, by piercing holes and reorganizing the matrix (Gaggioli et al. 2007). In the original study, α3β1 and α5β1 integrins were needed for vulval integrin-dependent CAF cell migration in an artificial-mixed collagen I/laminin-111 matrix. A separate study of pancreatic ductal adenocarcinoma (PDAC) CAFs has demonstrated that α3β1 integrin in this experimental system could bind to laminin-332 to support CAF differentiation and maintenance and to promote PDAC cancer cell invasion (Cavaco et al. 2018).

We have below assembled and updated knowledge related to the role of CAFs in pancreatic, lung, and breast cancers. In this updated version, we have highlighted the importance of integrin-mediated cell–ECM interactions in the TME, including potential roles of these interactions in chemoresistance which as another aspect of TME biology where the role of integrins is severely underexplored.

11.3 Pancreatic Cancer

11.3.1 CAF Heterogeneity in Pancreatic Cancer

An increasing number of studies highlights the significance of the endogenous stroma as source of CAFs. The developmental origin of the tumor stroma varies depending on the tissue. In pancreatic cancer, resident fibroblasts and stellate cells are thought to be the two main stromal origins of CAFs. Although hepatic stellate cells (HSCs) have been found to be of mesothelial origin (Friedman 2000; Asahina et al. 2011), the origin of pancreatic stellate cells (PSCs) is still debated. PSCs have been suggested to have a neuroectodermal origin, but more recent data suggest that PSCs may arise from pancreatic mesenchyme (Harari et al. 2019; Wu et al. 2020). In the resting state, HSCs and PSCs can easily be recognized by storing lipid droplets. However, this does not remain an identifying criterion in PSC-derived CAFs though this is still an area of active research. A stromal cell population that is receiving attention in the pancreas are Gli1-positive cells. In a detailed study where Gli1 expression in pancreas was compared with a subpopulation of stromal cells expressing Hoxb6, only the Gli^+ cells expanded under fibrotic conditions suggesting that one should be careful when analyzing stromal cell subpopulations, since

dynamic changes during disease progression take place (Garcia et al. 2020). Another study suggests that only 10–15% of PDAC CAFs are derived from PSCs (Helms et al. 2021). In the literature, the nomenclature for the nonepithelial fibroblast-like cells in pancreas is confusing. It is unfortunate when the PSCs are called fibroblasts, whereas with the current nomenclature, it is unproblematic to call PSC-derived activated cells CAFs. When in doubt about the nature of nonepithelial cells in a tissue, the term "stromal cells" seems most appropriate.

Öhlund and colleagues (Ohlund et al. 2017) were among the first to characterize two major types of CAFs in the transgenic $LSLS^{KrasG12D/+}$; $Trp53^{R172H/+}$; $Pdx^{cre/+}$ (KPC) mouse model of PDAC and named these myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAF). myCAFs are located peritumorally, express high levels of α-SMA and fibroblasts activation protein (FAP), and require cell–cell contact to be competent to differentiate into this subtype. In contrast, iCAFs are present at farther distance from tumor cells and express lower levels of FAP and α-SMA and secrete inflammatory cytokines such as IL-6. The authors furthermore convincingly show that CAFs could alternate from myCAFs to iCAFs and inversely, in a dynamic manner. Later studies identified a third subset of mouse CAFs characterized by expression of major histocompatibility complex (MHC) class II antigens suggested to be active in T-cell activation (Elyada et al. 2019). This third population, which is demonstrated to share transcriptional profile with mesothelial-like cells has been named antigen-presenting fibroblasts (apCAF).

In a separate study by Dominguez et al., careful comparisons of CAF subtypes in mouse PDAC tumor models and human PDAC tissues resulted in several noteworthy observations (Dominguez et al. 2020). In mouse PDAC stroma, five subclusters of stromal cells were identified, all characterized by podoplanin (PDPN) expression, with three of the subclusters corresponding to myCAFs, iCAFS, and apCAFs, respectively, and two subclusters with characteristics of normal fibroblasts. The apCAF subcluster was noted to have a mesothelial-like transcriptional profile. Comparison of the mouse and human pancreatic tumor tissues suggested the absence of specialized antigen-presenting apCAFs in human PDAC TME. Instead, the two major subsets of CAFs, iCAFs and myCAFs, expressed CD74 and HLA-DRA and were implicated in antigen presentation. Interestingly, only one integrin gene, *ITGA11*, was profiled to be highly expressed on the myCAFs. When comparing the data from PDAC TME tissues with other human tumor types, a "TGF-β CAF signature" consisting of an 11-gene-signature was identified. These data reveal a potential functional difference between human and mouse CAFs. From an integrin perspective, there are few integrins present in these gene lists, an observation made not only from the study by Dominguez et al. (Dominguez et al. 2020), but also from other studies. This is indeed intriguing since integrin are known to be central in matrix remodeling and integration of paracrine signaling. We can think of a few reasons to explain these observations:

1. In transcriptional analysis of a mixed cell population, potential changes in integrin regulation in an individual cell population may not be easily detected (up in some cell types, down in some other cell types, possibly neutralizing each

other). To analyze changes in integrins might require synchronized, more pure cell populations or using single cell profiling.

2. Integrins are known to be sensitive to growth factor levels, and TGF-β in general being a strong regulator of integrin levels. A failure to detect changes in integrin regulation in fairly homogenous sorted cell populations might thus reflect poor sensitivity of the assay for low abundant proteins (including membrane proteins such as integrins). Even though membrane protein levels are of low abundance in comparison to many intracellular and secreted proteins, they are often central in cell regulatory events. We predict that future analysis of molecular mechanism with more sensitive analyses will clarify the role of integrins in the TME. In the study of Dominguez et al., the presence ITGA11 on CAFs with a TGF-β regulated signature is interesting since the ITGA11 promoter contains a SMAD-binding element (Lu et al. 2006, 2010).

In the majority of models involving tumor-stroma interactions, detailed studies suggest that the stroma is tumor supportive (Alexander and Cukierman 2016; Han et al. 2015). In molecular terms, this corresponds to various types of cross-talk between tumor and stromal cells including both paracrine and integrin-mediated signaling. In pancreatic cancer, the stroma has been described to support tumor growth and metastasis as well as the development of chemoresistance (Pan et al. 2015). Other studies suggest that stroma might act as a restraining barrier preventing tumor expansion and tumor spread.

Several studies involving TME will have to be revisited in regard with the new knowledge about CAF heterogeneity.

New data generated in more targeted approaches to CAF subsets support data from broadly cited study from Ozdemir and colleagues, suggesting that conditional deletion of α-SMA-expressing fibroblasts in experimental PDAC mouse model aggravates tumor outcome (Ozdemir et al. 2014). In this study, the α-SMA-thymidine kinase mouse was crossed with two different models of PDAC, namely the KPC mouse and $Ptf1a^{cre/+}$; $Kras^{Gt2D/+}$;$TGFbr2^{flox/flox}$ (PKT) mouse, allowing depletion of α-SMA-expressing cells. This approach led to decreased number of myofibroblasts and ensued more invasive, undifferentiated, and necrotic tumors. The cell population targeted in this approach was most likely the CAF subpopulation we now call myCAFs, but also α-SMA positive cells in the tumor vasculature are affected. In a more recent study, an advanced PDAC mouse model was used to analyze the effects of collagen I deletion in α-SMA-expressing cells and in the context of TME (Chen et al. 2021). The results indicate that reduced collagen I expression results in more proliferative tumor cells, supporting the concept that the collagen I-producing myCAFs are tumor re-restraining. But also in this model, there are caveats, since not only myCAF-sythesized collagen I was targeted, but also the CAF-independent collagen I that could also contribute to the observed phenotype. Interestingly, Chen et al. propose that myCAFs lacking collagen I synthesis stimulate the PDAC cells to secrete CXCL5 which in turn attracts neutrophils contributing to an immunosuppressive environment, which further explains the tumor-restraining phenotype. These results agree with recent data from a study showing a correlation between collagen content and disease severeness (Jiang et al. 2020). PDAC patients

with more desmoplasia had a better prognosis, which led the authors to inhibit LOXL2 (lysyl oxidase-like 2) activity in a mouse tumor model. Their data thus also support the concept that a collagen matrix restrains tumor growth in the PDAC context.

Curiously, focal adhesion kinase (FAK) inhibition in tumor cells appears to reduce tumor desmoplasia, but without leading to increased tumor aggressiveness or tumor spread (Jiang et al. 2016). This study clearly demonstrates that the molecular mechanism whereby ECM amounts and properties are changed can have different effects on tumorigenesis (cf. collagen deletion in myCAFs above). Similar findings, where cell type specific features determine molecular mechanisms, have been observed for the role of αvβ6 in experimental PDAC models (Reader et al. 2019). Great care thus must be taken when analyzing different models where knowledge about, for example, receptor repertoires and mutations in signaling pathways must be considered.

11.3.2 Tumor Cell Integrins in Pancreatic Cancer

FAK plays an important role in integrin signaling. The importance of integrin signaling in PDAC is indirectly indicated in studies in which FAK inhibitors failed to inhibit tumor angiogenesis, apoptosis, or necrosis but decreased tumor size and the populations of CAFs and tumor-associated macrophages (TAMs) within tumors (Stokes et al. 2011). This work is further supported by studies in which blocking FAK in PDAC models where a FAK inhibitor reduced tumor cell proliferation, the secretion of pro-fibrogenic paracrine signals and fibrosis in the TME. An important finding in this study is that blocking FAK and reducing ECM of TME did not lead to increased PDAC tumor cell growth in these experimental systems, but instead had the opposite effect. This is thus in contrast to the recent study by Chen et al. (2021) in which reducing collagen I synthesis in myCAFs increased tumor invasiveness. Obviously, conditional deletion of FAK in different cell types would be required to understand the mechanism of how FAK works in the context of integrin adhesion and paracrine signaling.

The collagen-binding integrins α1β1 and α2β1 are reported to be expressed on PDAC cells. One study suggests that α1β1 can be used as a premalignant biomarker in PDAC, and it was also shown to promote metastatic potential of cancer cells and induce therapy resistance (Gharibi et al. 2017). α2β1 is expressed also in blood vessels and immune cells, and in epithelial and endothelial cells, it binds the basement membrane collagen IV (Barczyk et al. 2010). In this context, it is interesting to note that the secreted glycoprotein galectin-3, which is often found upregulated in PDAC, can, when secreted, form networks in the ECM which specifically influence α2β1 integrin to impede integrin clustering and avidity (Friedrichs et al. 2008). This could be one of many mechanisms by which TME composition affects integrin activity in different cell types expressing α2β1 in PDAC tumors (Table 11.1).

Table 11.1 Role of integrins in desmoplastic tumors

Tumor type	Cell type	Experimental model	Integrin/ integrin subunit	Role	Effects tumor microenvironment
Pancreatic ductal adenocarcinoma (PDAC)	Tumor cells	PDAC/CAF spheroids, orthotopic PDAC models in nude mice	αvβ5	Regulated by TGF-β in TME. Regulates tumor growth and metastasis, role in chemoresistance (Hurtado de Mendoza et al. 2021)	ND
	Tumor cells	PDAC cell lines in vitro, KDC mouse model in vivo	αvβ6	TGF-β-activating, pro-migratory via Eps8, acting as switch between migratory and TGF-β activating-role (Tod et al. 2017). Promotes tumor growth, and tumor desmoplasia in KDC mice (Reader et al. 2019)	Indirect via activation of TGF-β ++
	Tumor cells	Human tissues, PANC-1 cells in vitro	α1	Pre-malignant biomarker, promotes metastasis, chemoresistance (Gharibi et al. 2017)	ND
	Tumor cells	Human xenografts in mice, PDAC cells in vitro	α2	Promots growth and chemoresistance (Semba et al. 2004; Dangi-Garimella et al. 2011)	ND
	Tumor cells	In vitro studies	α5	RAB5A-mediated endocytosis of α5β1 to promote migration (He et al. 2019)	ND
	CAFs	In vitro and in vivo studies.	αvβ5	Regulates α5 integrin levels and activity by regulating endocytosis (Franco-Barraza et al. 2017)	++
	CAFs	In vitro studies	α3	Supports maintenance of CAFs and stimulates PDAC cell growth (Cavaco et al. 2018)	+
	CAFs	PDAC subcutaneous tumors in mouse models in vivo	α5	Stimulates tumor growth and tumor desmoplasia, affect chemotherapy efficacy (Kuninty et al. 2019)	+++

(continued)

Table 11.1 (continued)

Tumor type	Cell type	Experimental model	Integrin/ integrin subunit	Role	Effects tumor microenvironment
	CAFs	Cultured PSC. Primary and immortalized PDAC CAFs in vitro	α11	Present on PDAC CAFs in vitro and in vivo (Zeltz et al. 2019). Identified on human PDAC myCAFs by scRNA-seq as part of TGF-β CAF signature (Dominguez et al. 2020). Promotes PSC myofibroblast activation and cell migration in vitro (Schnittert et al. 2019). Functional in vivo data lacking	+++
Breast cancer	Tumor cells		αvβ6	Activate TGF-β, SOX4 activation and tumor cell resistance to cytotoxic T cells (Bagati et al. 2021). Expressed in subset myoepithelial cells (Allen et al. 2014)	+++, CAF activation and ECM production
	Tumor cells	Itgb1$^{-/-}$//Itgb3$^{-/-}$ mouse model	β1/β3	In ERbB2-dependent breast cancer integrins form a complex with insulin receptors (IRs) and activate Akt/mTORC1 signaling axis to stimulate growth and metastasis (Huck et al. 2010; Bui et al. 2019)	ND
	Tumor cells		β3	Takes part in extracellular vesicle uptake, shown to influence clonal expansion of tumor cells (Fuentes et al. 2020)	ND
	Tumor cells		α3	Represses Reelin expression to promote invasion (Ndoye et al. 2021)	ND
	Tumor cells		α4	Tumor cell invasion (Kwon et al. 2020).	ND
	Tumor cells		α5	FN fibrillogenesis in a stiff matrix in a Mena-dependent manner, facilitating α4β1-mediated tumor spread (Berger et al. 2020). Mediates metastasis to bone and osteolytic lesions (Pantano et al. 2021)	++++

	CAFs	Ddr2$^{-/-}$ mouse model	β1 (collagen-binding integrins)	Activated by DDR2 (Bayer et al. 2019), leads to remodeled collagen matrix	+++
	CAFs	Spin90$^{-/-}$ mouse model	α4	FN fibrillogenesis (Kwon et al. 2020) in SPIN90 knockout model, increased fibrillar matrix	+++
	CAFs	PyMT//Itga11$^{-/-}$ mouse model	α11	CAF activation, collagen remodeling, tumor growth, metastasis, expressed in subset of myoepithelial cells (Zeltz et al. 2020; Smeland et al. 2020; Primac et al. 2019)	+++
Non-small cell lung cancer	Tumor cells	NSCLC tumor cells, co-culture fibroblasts	αvβ6	Activates TGF-β in CAFs to stimulate tumorigenesis (Eberlein et al. 2015)	+++
	Tumor cells	PC9 tumor cells, PDX mouse tumor models	β1	Erlotinib resistance (Kanda et al. 2013), CAFs in inflammatory TME niche produce collagen, binding to β1 integrins on cancer cells, induce cell proliferation and tumor relapse (Cho et al. 2020)	CAFs are the cells that stimulate cancer cell proliferation
	Tumor cells	PC9 tumor cells	α2	Erlotinib resistance (Kanda et al. 2013)	ND
	Tumor cells	PC9 tumor cells	α5	Erlotinib resistance (Kanda et al. 2013)	ND
	CAFs	Mouse models in vivo, human NSCLC CAFs in vitro, gene signature	α11	Regulates stromal stiffness and metastasis in vivo, CAF migration in vitro (Navab et al. 2016; Iwai et al. 2021)	+++

A collection of selected references has been assembled to illustrate importance of integrins in tumor cells and cancer-associated fibroblasts (CAFs). We have also noted if integrins are implied in affecting the tumor microenvironment
FN fibronectin, ND not determined

In PDAC, αvβ3 integrin on tumor cells interacts with CAF-produced osteopontin to stimulate EMT and cancer stem cell-like properties by regulating the expression of FOXM1 at the tumor–stroma interface (Cao et al. 2018). The CAFs that secrete osteopontin probably correspond to the myCAFs. A study of human colon cancer has identified interactions between a specific subset of CAFs at tumor stroma with osteopontin, which in turn was suggested to influence cancer stem cells (Lenos et al. 2018). These studies that show CAF-mediated signaling activity at the stroma–tumor interface is clearly distinct from CAF interactions elsewhere in the tumor (Costa et al. 2018; Ohlund et al. 2017).

Two other αv-integrins that are relevant in the pancreatic PDAC cells are αvβ5 and αvβ6. High level of β5 in PDAC cancer correlates with reduced survival (Uhlen et al. 2017). In a careful study using KPC mice, β5 on PDAC cells was shown to promote tumor progression and metastasis (Hurtado de Mendoza et al. 2021). Interestingly, CAFs were shown to stimulate β5 levels on cancer cells via TGF-β signaling, again attesting to the importance of bidirectional paracrine signaling involving the TME.

Integrin αvβ6 has been found to be upregulated in a majority of PDAC cases (Brown and Marshall 2019). It has been reported to promote invasion and metastasis and is also the principal epithelial activator of TGF-β via binding to LAP (latency-associated peptide) (Munger et al. 1999). In this role as activator of TGF-β, αvβ6 indirectly influences the TME by providing TGF-β to CAFs and thus contributes to ECM synthesis and matrix remodeling (Reader et al. 2019). TGF-β can also act as tumor suppressor, and thus targeting TGF-β-activating integrins can be problematic, which highlights the need to dissect molecular mechanisms of action of αvβ6 and TGF-β in different tumors, tumor types, and tumor stages (Ahmed et al. 2017). Hence, for TGF-β-activating integrins, it is thus critical to choose the right animal or cell model and be aware if the function of TGF-β in the given model is growth-supportive or growth-suppressive. It is furthermore interesting to note that in addition to reduced tumor growth, the antibody blockade of αvβ6 led to reduced tumor desmoplasia (Reader et al. 2019), lending evidence to the indirect effect of epithelial αvβ6 on the CAF-produced ECM in the TME (Brown and Marshall 2019).

11.3.3 CAF Integrins in Pancreatic Cancer

We have generated a novel function-blocking antibody against integrin α11 that inhibits CAF interaction with collagen I, collagen reorganization, and CAF migration in a spheroid-based PDAC model (Zeltz et al. 2019) (Fig. 11.2). Schnittert and colleagues suggested that α11β1 on PDAC CAFs is indeed the main integrin that can drive PDAC cell invasion in an hetero-spheroid model (Schnittert et al. 2019). This study was based on use of a commercial polyclonal antibody to α11 with limited ability to distinguish specific reactivity on different stromal cells. Although in vitro data demonstrated a role of α11β1 in stellate cell myofibroblast differentiation, the lack of markers that can unequivocally separate PSC/pericytes from fibroblasts

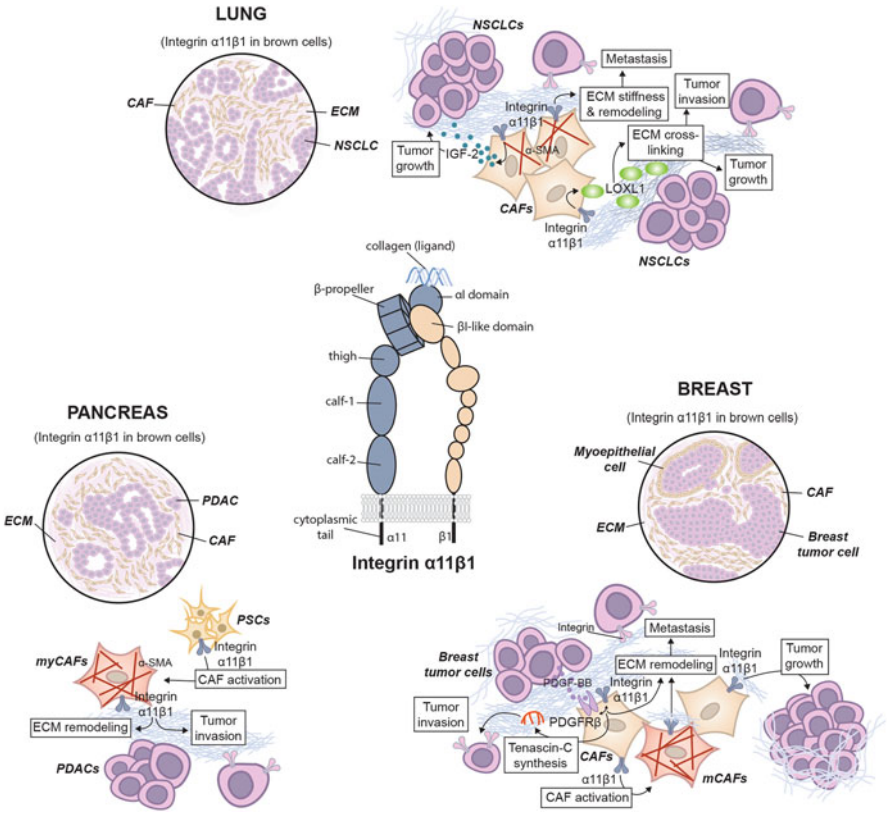

Fig. 11.2 Focus on integrin α11β1 in cancer. Integrin α11β1 is a collagen receptor expressed on a subpopulation of fibroblasts. The schematic summarizes its expression and functions in pancreatic, lung, and breast cancers. Integrin α11β1 is expressed on cancer-associated fibroblasts (CAFs) in the three cancer types and in myoepithelial cells surrounding duct in breast cancer. *Integrin α11β1 in lung tumor microenvironment (TME):* Integrin α11β1 expressed on CAFs controls ECM stiffness and remodeling and regulates the secretion of IGF-2, contributing to metastasis and tumor growth of nonsmall cell lung cancer (NSCLC), respectively. Moreover, integrin α11β1 modulates the expression of lysyl oxidase-like 1 (LOXL1), which is an ECM cross-linking enzyme implicated in tumor cell growth and invasion. *Integrin α11β1 in breast TME:* PDGF-BB is released by mammary tumor cells and binds to its receptor PDGFRβ on CAFs. PDGFRβ cross-talks with integrin α11β1 to support metastasis. Integrin α11β1 activate CAFs into myofibroblasts to remodel the extracellular matrix, which in turn affects tumor growth and metastasis. *Integrin α11β1 in pancreas TME:* TGF-β released in pancreatic ductal adenocarcinoma (PDAC) induces the formation of desmoplasia by CAFs. Integrin α11β1 in pancreatic stellate cells (PSCs) signals to differentiate into myofibroblastic CAFs (myCAFs). Integrin α11β1 controls ECM reorganization to mediate PDAC invasion

renders it difficult to judge on which cell type α11 is active in PDAC in an in vivo situation. Is it active on CAFs derived from PSC population or is it active on CAFs derived from a distinct fibroblast subpopulation? Whereas blockage of α11 on PSC in vitro inhibits myofibroblast differentiation, no blockage of α11 has so far been performed in a PDAC tumor model in vivo. We have confirmed that α11 is present

on CAFs in PDAC tumor stroma in vivo using the novel monoclonal α11 antibodies we have generated, but not in NG2 (Nerve/Glial antigen 2)-positive CAFs (Zeltz et al. 2019). We have also failed to detect α11β1 in cells with pericyte characteristics (Zeltz et al. 2019). Thus, the determination of the source of α11-expressing CAFs using cell lineage tracing will be crucial to further study the role of α11β1 in PDAC, both to define the CAF subset with relation to other markers and to test effect of blocking or deleting α11 in an in vivo model. Careful analysis of the data by Dominguez et al. reveals that in PDAC, *ITGA11* is transcriptionally active in a subcluster of fibroblasts which correspond to a subset of myCAFs (Dominguez et al. 2020). In the pancreas, it is thus possible that α11-positive myCAFs are both of PSC and fibroblast origin. When analyzing integrins of different subsets of CAFs, it is important to remember that integrin upregulation in cell culture is a well-known fact. Integrin α11 is readily upregulated on cells in vitro that never have been found to express α11 in vivo. This might actually be the case for both mouse liver and pancreas where we have failed to detect α11 expression in either HSC or PSC in vivo, but α11 is clearly detected on both stellate cell subsets during in vitro culture (Martin et al. 2016; Schnittert et al. 2019).

Interestingly, PDAC cells can migrate on FN deposited on the CAF surface protrusions in a 3D collagen matrix using their α5β1 integrin (Miyazaki et al. 2019). It has also been demonstrated that α5β1 integrin endocytosis is regulated by αvβ5, indirectly affecting CAF activation (Franco-Barraza et al. 2017). In colon cancer, αvβ3 integrin expressed on CAF, together with integrin α5β1, contributes to FN fibrillogenesis and tumor cell invasion (Attieh et al. 2017). It will be interesting to establish whether αvβ3 has such a role also in PDAC CAFs.

Thus, present data imply that α5β1 and α11β1 integrins are important CAF receptors involved in matrix assembly and reorganization engaged in tumor cell growth and migration. Integrins αvβ3 and αvβ5 have both been found to assist or regulate integrin α5β1 activity while little is known about crosstalk of α11β1 with other integrins in pancreatic cancer. In tissue fibrosis, αvβ1 integrin has been demonstrated to orchestrate the activation of TGF-β on myofibroblasts and it will be thus interesting to have a comparable role on (a) particular PDAC CAF subtype (s). In addition, it is important to show which CAF subpopulations in PDAC are tumor-supportive and which are tumor-suppressive and integrate the relative contribution of specific CAF integrins in the paracrine signaling of iCAFs and myCAFs. In parallel, it will also be essential to understand whether different CAF subtypes hold prognostic value and have different functional properties (Table 11.1).

11.3.4 CAF/Integrin-Mediated Chemoresistance in Pancreatic Cancer

Integrin α1β1 is a receptor for the basement membrane collagen IV, and as already mentioned, one study suggests that it is associated with chemotherapy resistance in PDAC (Gharibi et al. 2017). The authors used mainly human PDAC cell lines to

demonstrate that α1β1 is upregulated and coregulated with other TGF-β-responsive genes during EMT in PDAC cells. Interestingly, knocking down ITGA1 sensitized mesenchymal-like PDAC cells to the cytotoxic nucleoside analog gemcitabine.

αvβ5 is present on both PDAC cancer cells and stromal cells and is potentially a useful target to reduce desmoplasia. A recent study documented in vivo evidence for the role of αvβ5 in KPC mice and showed that iRGD (internalizing RGD peptides) targeting αvβ5 potentiated the effect of chemotherapy in this model (Hurtado de Mendoza et al. 2021). Although αvβ6 is present on PDAC cells, its central role in TGF-β activation indirectly affects CAF-mediated ECM synthesis and is therefore also an attractive target molecule that can be used to overcome CAF-mediated chemoresistance (Reader et al. 2019; Moore et al. 2020; Meecham and Marshall 2021). Tumor-associated macrophages and CAFs both contribute to TME-mediated chemoresistance in orthotopic genetic animal models like the KPC mouse and involvement of insulin-like growth factors (IGFs) in these processes has been suggested (Ireland et al. 2016; Ireland and Mielgo 2018). In this context, it would be interesting to determine the effect of IGFs on the integrin repertoire and on CAF-immune cell cross-talk.

11.4 Breast Cancer

11.4.1 CAF Heterogeneity in Breast Cancer

Breast is a complex organ, which undergoes hormonally regulated changes. In normal mouse mammary glands, the stroma largely determines the glandular epithelium development. Two subsets of fibroblasts have been identified in human mammary gland, lobular ($CD105^{high}/CD26^{low}$) and interlobular ($CD105^{low}/CD26^{high}$) fibroblasts (Morsing et al. 2016). CAF heterogeneity in breast cancer stroma has been observed both in human patient samples (Costa et al. 2018) and in mouse models (Bartoschek et al. 2018; Raz et al. 2018). In the human breast cancer, four different subsets of CAFs, CAFS1–CAFS4, have been identified, based on the expression profiles of six different markers (Costa et al. 2018). Notably, two of the subsets, CAF-S1 and CAF-S4 express high levels of α-SMA, but only CAF-S1 expresses fair amounts of FAP. CAF-S1 is found close to the tumor, attracts T-cells and contributes to immunosuppression. The immunosuppressive function of CAF-S1 partly depends on dipeptidylpeptidase 4 (DPP4, also known as CD26) and DPP4-mediated cleavage of CXCL10, leading to a reduction in T-cell recruitment to the tumor. The careful study by Costa et al. also indicates that within the four CAF subclasses there is probably even more heterogeneity. The CAF-S1 displays similar characteristics to those in myCAF population in pancreatic cancer (Costa et al. 2018).

Using single-cell RNA sequencing (scRNA-seq), the issue of CAF heterogeneity was independently addressed in the MMTV-PyMT (hereafter, PyMT) mouse model of mammary cancer at late stages of tumor progression (Bartoschek et al. 2018). This

study identified four transcriptionally distinct subsets of CAFs with different functionalities and biophysical properties. They were termed as vCAFs (vascular CAFs), mCAFs (matrix CAFs), cCAFs (cell cycle CAFs), and dCAFs (developmental CAFs) and presented distinct spatial location within the tumor parenchyma. vCAFs were shown to originate from the perivascular compartment with cCAFs being a segment of proliferative vCAFs. Conversely, mCAFs mostly derived from resident fibroblasts, while dCAFs seemed to originate from the malignant epithelial compartment via EMT. Interestingly, PDGFRα was specifically expressed by mCAFs, whereas PDGFRβ was expressed by most CAF subsets, with exception of dCAFs. In contrast to the previously mentioned study (Costa et al. 2018), FAP and α-SMA markers were not specifically associated with a distinct subset of murine CAFs, but rather displayed a salt-and-pepper expression pattern in all four CAF subpopulations. These discrepancies are presumably related to differences between species, breast cancer subtypes and stages, and to detection methodologies.

As already mentioned, cell lineage tracing in PyMT tumors demonstrated the contribution of mesenchymal, nonhematopoietic bone marrow cells to a PDGFRα$^-$ and clusterin$^+$ CAF subpopulation (Raz et al. 2018). In vitro, bidirectional paracrine signaling between tumor cells and this CAF subpopulation had effects on both tumor cells and the CAFs. Clusterin, which has pleiotropic effects including stimulation of endothelial cell proliferation, was suggested to promote tumor growth mainly via enhancing angiogenesis. This further highlights the complexity of fibroblast heterogeneity in breast cancer and suggests that this challenging issue requires additional investigation with regards to biomarker expression, spatial localization, and functionality of CAFs in all the subtypes and stages of breast cancers.

11.4.2 Tumor Cell Integrins in Breast Cancer

The literature on integrins in breast cancer is quite comprehensive and until recently has focused on integrins on the tumor cells. We will summarize some of this data below before going to the relatively scarce literature on CAF integrins in the TME. Detailed studies reveal a redundancy of β1 and β3 integrins to promote ErbB2-driven breast cancer (Bui et al. 2019). When β1 integrin is inactivated in epithelial cells in an ErbB2-driven mouse mammary cancer model, the resulting phenotype is a modest delay in tumor induction, which has been suggested to be due to β3 integrin compensation (Huck et al. 2010). In a double *Itgb1/Itgb3* knockout model of ErbB2-dependent breast cancer, the mice demonstrate a more severe delay in tumor onset, thus supporting the previous suggestion that mild tumor phenotype in *Itgb1* null background was due to integrin β3 compensation. Mechanistically, it was shown that in wild-type tumor cells, integrins can form a complex with insulin receptors (IRs) and activate Akt/mTORC1 signaling axis to stimulate tumor growth and metastasis (Bui et al. 2019). IRs are degraded in soft matrices, but the integrin-mediated ECM stiffness seems to stabilize IR levels at the cell surface and increase cell signaling. Interestingly, β3 integrins on triple negative human MDA-MB-231 breast cancer

cells have been demonstrated to be needed in a totally different context, namely for extracellular vesicle uptake allowing intercellular communication affecting metastasis and clonal growth capacity (Fuentes et al. 2020).

Regarding specific integrin α-chains and integrin heterodimers which have been implied on breast cancer cells, we have collected some recent data. In one example, α5 integrin chain is expressed in breast cancer cells and interacts with the intracellular an actin regulating protein called Mena. Increased stiffness and epidermal growth factor receptor 1 (EGFR) activation upregulate expression of Mena in breast cancer cells (Berger et al. 2020). Mena binds directly to α5 cytoplasmic tail to enhance α5-mediated outside-in integrin signaling and formation of FN-containing fibrillar adhesions (Gupton et al. 2012). The increased Mena levels stimulate α5β1 integrin-dependent EDA–FN organization to facilitate matrix metalloprotease (MMP)-dependent integrin α4β1- and α9β1-mediated breast cancer cell invasion. Independent studies suggest that α5β1 mediates metastasis to bone and osteolytic lesions (Pantano et al. 2021).

αvβ6 in breast cancer is involved in activating TGF-β with effects on both the tumor cells and CAFs in the TME such as increased collagen production and collagen remodeling by CAFs (Raab-Westphal et al. 2017; Brown and Marshall 2019). In one study, induction of αvβ6 in myoepithelial cells in preinvasive ductal carcinoma in situ was suggested as biomarker of disease progression (Allen et al. 2014). An elegant study furthermore demonstrated downstream effects of TGF-β activation in tumor cells leading to SOX4 activation and tumor cell resistance to cytotoxic T cells (Bagati et al. 2021). In this model antibody inhibition of αvβ6 induced T cell-mediated immunity in immunotherapy-resistant tumor models.

In addition to integrins that bind interstitial matrix components during invasion, integrins that adhere tumor cells to basement membranes have also been studied. These include laminin-binding integrin α3β1, which has been shown to repress secretion of Reelin, a secreted glycoprotein that inhibits invasion, with the end-effect of promoting invasion (Ndoye et al. 2021). Another study shows that ablation of α3β1 promotes tumor progression and invasion in Erb2-driven mouse mammary cancer model and in HER2-overexpressing human breast cancer cells, but not in triple-negative cancer cells (Ramovs et al. 2019). It should be also noted that α6β1 is a common marker of breast cancer stem cells (CSCs) and integrin-ECM interactions and FAK signaling are essential for maintenance of CSC pool and tumor progression (Guan 2010; Krebsbach and Villa-Diaz 2017).

11.4.3 CAF Integrins in Breast Cancer

One of the most notable features of tumor stroma interactions in breast cancer is the desmoplastic reaction. Extensive desmoplastic reaction in normal breast tissue in form of mammographic density is strongly correlated to an increased risk of breast cancer development and has been proposed as a diagnostic and prognostic marker. The ECM composition and architecture associated with this fibrotic reaction emerge

from an intimate crosstalk between fibroblasts and epithelial cells in breast tissues. Desmoplasia has also been linked to increased activation of integrins in breast cancer. In a breast cancer model, tumor cell-secreted LOXL2 activates fibroblasts and promotes the expression of α-SMA in a FAK-dependent manner (Barker et al. 2013). A previous landmark paper has demonstrated that increased tumor stroma stiffness promotes tumor progression by β1 integrin signaling in a FAK and Rho-signaling dependent manner (Levental et al. 2009). Likewise, in a mouse model of breast cancer, FAK inhibition decreased tumor growth and reduced infiltration of leukocytes and macrophages (Walsh et al. 2010; Wendt and Schiemann 2009). Together, these studies support the notion that β1 integrin and FAK sustains the pro-tumor functions of CAFs. The cooperation between integrins and receptor tyrosine kinase (RTKs) in tumor and stromal cells regulates cell invasion during metastatic dissemination in breast cancer. In this context, we have recently shown that stromal integrin α11 displays a pro-tumorigenic and pro-metastatic activity in breast cancer and strongly associates with a PDGFRβ+ CAF subset (Primac et al. 2019) (Fig. 11.2). Integrin α11 expression is strongly upregulated in the stromal compartment during mammary tumor progression. Histological analyses revealed a significant association between integrin α11 and PDGFRβ, both in human breast cancer samples and in the preclinical transgenic PyMT model. Among several tested stromal markers (PDGFRα, PDGFRβ, α-SMA, FAP, FSP1, and NG2), this collagen-binding integrin was mostly associated with a PDGFRβ$^+$ CAF subpopulation at late stages of tumorigenesis. As both integrin α11 and PDGFRβ are well-known regulators of ECM, it is plausible that the identified CAF subset overlaps with myCAF subpopulation described in the aforementioned study (Bartoschek et al. 2018). Indeed, genetic ablation of integrin α11 in the PyMT model drastically reduced not only tumor growth and metastasis, but also the desmoplastic reaction in these tumors, further highlighting the contribution of this specific α11$^+$ CAF subset to tumor progression through ECM regulation. This is further supported by the fact that mCAFs are thought to derive from resident fibroblasts, as well as integrin α11/PDGFRβ$^+$ CAFs. Mechanistically, this study revealed that integrin α11/PDGFRβ crosstalk in CAFs endows mammary cancer cells with pro-invasive features through the deposition of tenascin-C. Tenascin-C was strongly expressed by the same subset of CAFs expressing integrin α11 and PDGFRβ in the late stage PyMT tumors, as well as in clinical samples of invasive breast cancer. Overall, this study discloses an example of a collaborative crosstalk between an integrin and a RTK in CAFs, which acts as a driver of tumor invasiveness in breast cancer. Similar molecular partnerships have been previously reported, although not on CAFs. Indeed, microenvironment-induced c-Met/β1 integrin complex formation was shown to sustain breast cancer metastasis via the promotion of c-Met phosphorylation, as well as an increase of integrin affinity for FN on the tumor cells (Jahangiri et al. 2017). Further examples of cooperation between integrins and growth factor receptors in the context of cancer are thoroughly discussed in previous reviews (Ivaska and Heino 2011; Schnittert et al. 2018). It is worth noting that α2 integrin subunit, which heterodimerizes with β1 subunit to form another fibrillar collagen-binding integrin, exerts opposite functions to α11β1 in a related mouse

mammary cancer model (Table 11.1). In contrast to α11 integrin chain, integrin α2 chain is expressed not only by CAFs, but also by tumor cells and other stromal cells. Furthermore, unlike integrin α11, the α2 subunit is downregulated in human breast cancer and suppresses metastasis in vivo (Ramirez et al. 2011). Hence, α2-deficient MMTV-neu mice display increased metastasis, which is suggested to result from the increased capacity of tumor cells to intravasate into the bloodstream.

Discoidin domain receptor 2 (DDR2) is a cell surface tyrosine kinase activated by collagens (Multhaupt et al. 2016). The functions of DDR2 in both CAFs and mammary carcinoma cells have been studied by performing global and tumor cell specific deletion of Ddr2 in mice (Corsa et al. 2016). Global deletion of Ddr2 does not affect primary tumor growth but results in reduced metastasis. Closer examination reveals that $Ddr2^{-/-}$ stroma contains reduced amounts of fibrillar collagen, with reduced diameter and impaired organization, suggesting critical Ddr2 functions in CAFs. A more recent analysis of DDR2 in the breast cancer stroma suggests that the DDR2 effect is mediated via β1 integrins and the role of DDR2 is thus via inside out signaling to activate the integrins taking part in collagen remodeling (Bayer et al. 2019). Since the cross-talk between β1 integrins and DDR receptors supposedly is not restricted to the TME, it is possible that integrins, together with DDRs, are involved in tumor cell invasion and metastasis (Xu et al. 2012). In support of a role of DDR2 in metastasis, its small molecule inhibitor WRG-28 inhibits tumor cell–TME interactions and tumor cell invasion (Grither and Longmore 2018). It will be interesting to sort out a possible cooperation of DDR2 and integrins in tumor metastasis (cancer cell integrins) and collagen reorganization (CAF integrins) and if such a link exists, identify the specific integrin(s) involved.

In summary, an important cooperation between integrin and RTKs is detected in breast cancer CAFs, raising several interesting questions. Future studies will for example determine if one and the same integrin can cooperate with different RTKs in different tumor stroma contexts or if the cooperation is integrin-specific and limited to some kinases. This also extends to mechanism of TME-mediated chemoresistance where data are emerging on the role of integrins in mediating chemoresistance to drugs targeting RTKs (Cruz da Silva et al. 2019). These mechanisms have mainly been described in cancer cells, but similar mechanisms are likely to operate in CAFs. Finally, the role of αv integrins (TGF-β activating mechanisms), α5β1 integrin and FN matrix assembly, and relation to the collagen remodeling α11β1 integrin will be important to study more in detail in breast cancer, just as in other desmoplastic tumor types.

11.4.4 CAF-Mediated Chemoresistance in Breast Cancer

CSCs are largely responsible for the continuous tumor growth and development of drug resistance, and many different signaling pathways contribute to these processes. Some of these pathways involving CAFs are highlighted below. For example, Hedgehog (Hh) ligand activity is detected in one third of triple negative breast

cancer (TNBC). In an animal model of TNBC with high Hh level, an upregulation of fibroblast growth factor 5 (FGF5) and an increased collagen remodeling activity was observed in CAFs (Cazet et al. 2018). The increased concentration of remodeled collagen at tumor–stroma interface correlated with increased pFAK levels as well as increased number of CSCs at the tumor–stroma interface. In this breast cancer model, treatment with an inhibitor of the Hh receptor Smoothened (SMOi) sensitized mice to chemotherapy. It will be interesting to determine what specific integrins are present and mediate the increased pFAK at the tumor–stroma interface.

Studies in breast cancer cell lines offer additional details as to how CSC formation via EMT may occur. Snail1, which is a central transcription factor in EMT, is an unstable protein that is ubiquinated. In experiments performed by Lambies et al., deubiquitination by a specific ubiquitinase (USP27X) contributes to Snail1 stability in turn contributing to increased EMT, increased numbers of CSCs and chemoresistance (Lambies et al. 2018). In addition, Snail1 stabilization in CAFs contributes to increased CAF activation. It will be interesting to see whether CAF Snail1 in this context integrates with integrin-dependent mechanoregulated signaling. Such mechanoregulation in myofibroblasts involving Snail1 has been shown to contribute to fibrosis and to depend on both YAP/TAZ and myocardin-related transcription factors (MRTFs) (Zhang et al. 2016).

In an impressive study of chemo-resistant patients of breast cancer, various CAF subsets were identified (Su et al. 2018). The $CD10^{+}GPR77^{+}$ CAF subset was found to sustain cancer stemness and to promote tumor resistance. Furthermore, targeting this subset of CAFs using antibodies to GPR77 restored chemosensitivity. The authors suggest that targeting the $CD10^{+}GPR77^{+}$ CAF subset could be an effective strategy against CSC-driven solid tumors. It will be interesting to relate this subset of CAFs with the different breast cancer CAF subsets identified by Costa et al., as well as with CAF subsets in other cancer forms (Costa et al. 2018). It will also be important to characterize the role of integrins in CAF-mediated chemoresistance in breast cancer. We believe this will be an important area of research in the coming years.

11.5 Lung Cancer

11.5.1 Lung Fibroblast Heterogeneity

The lung is also a complex organ where fibroblasts have several functions associated with normal lung function. Cell lineage tracing has been performed to identify and characterize the origin of fibroblast subsets (Li et al. 2018), but in mouse lung, single-cell transcriptional analysis has been even more instrumental and has resulted in the identification of five subsets of fibroblasts in healthy lung and six subsets in fibrotic lung, in addition to a mesothelial subtype (Xie et al. 2018). In normal lung, these were grouped as myofibroblasts (Acta2+), col3a1 matrix fibroblasts (Col3a1+; Itga8+), Col4a1 matrix fibroblasts (Col4a1+;dcn+), lipofibroblasts (Lp1+), and

mesenchymal progenitors (CD52+) (Xie et al. 2018). In the fibrotic lung, a distinct fibroblast cell type with high PDGFRβ expression, distinct from pericytes, was identified (Xie et al. 2018). In a separate study, the focus was on identifying subsets of cells involved in collagen synthesis in normal and diseased lung. The scRNA-seq analysis identified several subclusters within alveolar, adventitial, and peribronchial fibroblasts (Tsukui et al. 2020). Some major findings in this careful study include the identification of collagen XIII as a marker for lipofibroblasts, collagen triple helix repeat containing 1 (CTHRC1) as a marker for a subset of fibrotic fibroblasts in fibrosis predicted to be derived from endogenous alveolar fibroblasts and showing poor correlation between α-SMA-positive myofibroblasts and high collagen I production. Whereas studies of pancreatic and breast cancer tumors have started to unravel different subsets of CAFs, less detailed information is published on CAF heterogeneity in non-small cell lung carcinoma (NSCLC). In one interesting recent study, the influence of vascular adventitial fibroblasts on A549 lung cancer cells in a xenograft model indicated a higher tumor-promoting activity of the adventitial fibroblasts compared to non-vessel-associated lung fibroblasts (Hoshino et al. 2011). Microarray analysis further demonstrated a high level of PDPN expression in these adventitial fibroblasts, which in combination with other studies suggest a role for PDPN in tumorigenesis and lymph node metastasis. The study by Hoshini et al. in addition to demonstrating CAF heterogeneity in the lung tumor stroma suggests that a perivascular environment in lung constitutes a specific niche for tumor progression in the lung. PDPN expression has recently been shown to regulate β1 integrin levels in keratinocytes (Shibuya et al. 2018), whether this activity also applies to NSCLC CAFs remains to be determined. Studies using antibodies to detect FAP as a biomarker have also provided information, which clearly indicate the existence of CAF subsets in the lung (Kilvaer et al. 2018). Immunostaining of human NSCLC tumor sections in studies by Kilvaer et al. typically showed α-SMA and FAP expression on different CAFs, suggesting that in the lung, FAP is not highly expressed on myofibroblastic CAFs. The study also points out a major weakness with FAP antibodies in the context of the TME namely, FAP antibodies also immunostain macrophages. In tumors from NSCLC patients with high levels CD3+ and CD8+ T cells, high FAP levels on CAFs were associated with better prognosis. The latter finding indicates that FAP-directed therapy as a general antistroma therapy needs to be performed with great caution, and as already mentioned might not be suitable as a general antistroma therapy, but rather be suitable for a subset of tumors. The general expression pattern of CAF markers needs great attention in therapy situations. This has also been illustrated by experiments with FAP-directed immunotherapy where a side-effect of the tumor-directed treatment was cachexia, due to expression of FAP in muscle (Roberts et al. 2013). Finally, as yet another example of the complex events that take place in the TME, a recent study of a cohort of NSCLC patients identified glutamin-fructose-6-phosphate transaminase 2 (GFPT2) in CAFs as being responsible for increased glucose uptake and metabolic reprogramming in the TME (Zhang et al. 2018b).

11.5.2 Tumor Cell Integrins in Lung Cancer

As in other solid tumors, integrin αvβ6 stands out again as an integrin on cancer cells, which when activated will have effects on the TME. Careful coculture studies with NSCLC cells and fibroblasts demonstrate activation of fibroblasts and stimulation of cancer cell growth by a mechanism dependent on αvβ6-mediated TGF-β activation (Eberlein et al. 2015). Please also see chemoresistance section below for more integrins implicated in tumor cells.

11.5.3 CAF Integrins; The Role of Integrin α11β1 in Lung Cancer

In 2002, the Tsao laboratory published a list of six novel candidate genes for lung adenocarcinoma (obtained by comparing pooled RNA from tumors with normal lung RNA), which included *Lc-19, HABP2, CRYM, CP, COL11A1*, *and ITGA11* (Wang et al. 2002). In 2011, Navab et al. published a molecular signature for the NSCLC stroma (Navab et al. 2011). Matched pairs of normal lung fibroblasts and lung CAFs were isolated from 15 patients and their transcription profiles established. This effort resulted in identification of 46 differentially expressed genes in CAFs that formed a prognostic gene expression signature. Interestingly, six of the identified genes could be induced by TGF-β in normal fibroblasts, including the collagen receptor α11β1, identified in the original gene set from 2002. Comparison of these CAF genes with tumor stroma genes indicated a shared upregulation of four genes; ITGA11, THB2, COL11a1, and CRTHRC1. In the same study, analyses of epigenetic changes identified limited methylation changes in tested genes. Following the identification of α11 as one of a limited set of genes being upregulated in the stroma of experimental NSCLC tumors, the role of α11 in lung cancer was explored further. In xenograft models, coimplantation of NSCLC tumor cells with mouse embryonic fibroblasts lacking α11 greatly reduced NSCLC tumor growth (Zhu et al. 2007), which in this xenograft model was correlated to α11-dependent secretion of IGF-2. To functionally further test the potential contribution of α11 to CAF function in NSCLC, the integrin $\alpha11^{-/-}$ mouse strain has been very helpful (Popova et al. 2007). Analysis of NSCLS tumor growth in the $\alpha11^{-/-}$ mice demonstrated that absence of α11 in the stroma impeded NSCLC tumor growth (Navab et al. 2016) and metastasis. Analysis of tumor stroma demonstrated reduced organization of the collagen stroma and reduced tumor stiffness. Analysis of signaling status demonstrated reduced FAK and ERK phosphorylation in the tumor stroma from $\alpha11^{-/-}$ mice and reduced expression of α-SMA in the tumor stroma. Interestingly, integrin α11 has recently been shown to regulate the lysyl oxidase-like 1 expression in lung CAF to mediate NSCLC cell invasion and tumor growth (Zeltz et al. 2019) (Fig. 11.2). Protein–protein interaction network analysis in addition identified several interactions affected in the $\alpha11^{-/-}$ stroma and previously identified in CAF

differentiation and tumorigenesis, including latent transforming growth factor beta binding proteins 3 and 4 (LTBP3 and 4), WNT1-inducible signaling pathway protein 2 (WISP2), insulin-like growth factor binding protein 2 and 4 (IGFBP2 and 4), and syndecan-4. It will be interesting to determine whether, and how, these proteins take part in α11-mediated effects in CAFs and other types of stromal cells. That CAFs can regulate plasticity of lung cancer stemness via paracrine signaling was shown in experiments, which identified IGF-2 producing CAFs as inducers of Nanog expression in cancer cells and thus established that these lung CAFs constitute a supporting niche for cancer stemness (Chen et al. 2014). With regards to IGF-2, it is interesting to note that the integrin α11β-expressing fibroblasts in the xenograft model of lung cancer produce IGF-2 (Zhu et al. 2007), and part of the pro-tumorigenic action of α11β1 might thus be related to a stemness-stimulating activity.

11.5.4 Integrin-Mediated Chemoresistance in Lung Cancer

A study using PC9 cells demonstrated the involvement of β1 integrins and specifically α2β1 and α5β1 in Erlotinib chemoresistance by creating a Src bypass pathway to induce Akt signaling (Akt also activated by EGFR) (Kanda et al. 2013). A number of studies have addressed paracrine signaling at tumor–stroma interface. In the future, it will be important to correlate these with effects of dynamic changes in integrin repertoire in the TME cell types, including CAFs. Examples of such studies that would benefit from integrin analysis of TME-induced chemoresistance in the lung include a study where CAFs have been reported to produce IGF-2 as an inducer of the ABC transporter P-GP in A549 cells and to mediate drug resistance (Zhang et al. 2018a). In another study, lung adenocarcinoma CAFs treated with Cisplatin upregulated IL-11 and conferred chemoresistance to lung cancer cells by activating STAT3 antiapoptotic pathway (Tao et al. 2016). In agreement with these in vitro findings, patients with high levels of IL-11R display poor response to Cisplatin. Another careful study focused on the importance of activated CAFs in an inflammatory TME niche in relation to chemoresistance in dormant slow cycling cancer cells. In this study, combined in vitro experiments and in vivo mouse PDX (patient-derived xenograft) tumor model data suggest that CAFs produce collagen I that activate slow cycling cancer cells via β1 integrin and Src to become actively proliferating, leading to tumor relapse (Cho et al. 2020). Again, it would be interesting to identify the specific integrins involved in this mechanism.

11.6 Conclusions

With scRNA-seq-based transcriptional profiles of normal and fibrotic connective tissues accumulating, we will in the next few years see a more detailed stratification of fibroblast and CAF subtypes. The accumulated transcriptional profiles will have

to be verified at protein level and these validated profiles will in next step inspire the development of RNA and antibody tools that can be used as "biomarker fingerprints" for the increasing number of fibroblast- and CAF-subsets. These subset-specific profile tools will replace the old concept of "one fibroblast biomarker" and will contribute to a better understanding of the functionally specialized fibroblasts and fibroblast-derived cells. The emerging data suggesting an increasing heterogeneity of CAFs will most likely also be reflected in tissue- and subtype-specific modes of integrin functions. In this context, it will be important to characterize cell- and tissue-specific integrin functions in various CAFs in order to understand how paracrine and direct integrin-mediated interactions cooperate to regulate TME functions. Finally, we foresee that in the coming years, function-blocking integrin antibodies, integrin-based small molecules, as well as integrin antibody-drug conjugates have the potential to increase the efficacy of antifibrosis therapy strategies and to lead to development of improved novel immunotherapy protocols in tumor fibrosis.

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Chapter 12
Adipose Compounds in Breast Tumor Extracellular Matrix

Flavia Piccioni and Paola De Luca

Abstract Adipocytes constitute the major cell population of adipose tissue (AT), which in turn is an important breast tissue component. Many epidemiological studies support that AT plays a significant role in breast cancer (BCa) development and progression. In fact, several conditions associated with an increase of AT, such as obesity or waist-to-hip ratio, impact on BCa risk, and outcomes. Besides, in the last years, it has been demonstrated that invasive BCa cells modify adjacent adipocytes, becoming cancer-associated adipocytes (CAAs). These cells differ phenotypic and functionally from normal adipocytes, and secrete factors such as adipokines, inflammatory molecules, and microRNAs (miRNAs) that promote a pro-tumoral microenvironment. Moreover, CAAs can regulate processes such as fat distribution, insulin secretion, energy consumption, and inflammatory reactions. Importantly, studies have revealed that a cyclic crosstalk between adipocytes and BCa cells is established, from which breast tumor feeds back in order to proliferate, invade, and metastasize. Additionally, the altered AT represents a barrier to resistance for BCa treatment. Thus, therapies targeting AT in the context of BCa are currently the subject of extensive study. In this chapter, we will focus on the signaling molecules and the feedback that is established between BCa cells and AT, which allow BCa progression.

Abbreviations

AKT	AKT serine/threonine kinase
AMPK	Protein kinase AMP-activated catalytic subunit alpha 1
AT	Adipose tissue
ATX	Autotaxin
BAT	Brown adipose tissue

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BCa	Breast cancer
BMI	Body mass index
C/EBPα	CCAAT/enhancer-binding protein α
CAAs	Cancer associated adipocytes
CAFs	Cancer associated fibroblast
CAP1	Adenylyl cyclase-associated protein 1
CCL2	Chemokine (C–C motif) ligand 2
CCL5	Chemokine (C–C motif) ligand 5
CCR2	C–C motif chemokine receptor 2
CCR4	C–C motif chemokine receptor 4
CCR5	C–C motif chemokine receptor 5
CSCs	Cancer stem cells
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ER−	Estrogen receptor negative
ER	Estrogen receptor
ER+	Estrogen receptor positive
FABP4	Fatty-acid binding protein 4
FAs	Fatty acids
FFAs	Free fatty acids
FOXP4	Forkhead box P4
GLUT4	Glucose transporter type 4
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IGF	Insulin growth factor
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IL-1	Interleukin-1
IL-1R	Interleukin-1 receptor
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
JAK	Janus kinase
JAK2	Janus kinase 2
LDL	Low density lipoproteins
LPA	Lysophosphatidic acid
M1	Macrophage type 1
MCP-1	Monocyte chemoattractant protein-1
MCT1	MCTS1 re-initiation and release factor
MCT4	MCTS4 re-initiation and release factor
MeS	Metabolic syndrome
miRNAs	microRNAs
MMP11	Stromelysin-3
MMPs	Matrix metalloproteases

mTOR	Mechanistic target of rapamycin kinase
MVP	Major vault protein
NF-κB	Nuclear factor kappa-B
NGF	Nerve growth factor
NOTCH1	Notch receptor 1
ObR	Leptin receptor
p16INK4A	CDKN2A, cyclin dependent kinase inhibitor 2A
PAT	Pink adipose tissue
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPARγ	Peroxisome proliferator-activated receptor-γ
RANTES	Chemokine (C–C motif) ligand 5
S100B	S100 calcium binding protein B
SFRP1	Secreted Frizzled Related Protein 1
SPP1	Secreted phosphoprotein 1
STAT3	Signal transducer and activator of transcription 3
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor-alpha
UCP-1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WAT	White adipose tissue
Wnt3a	Wnt family member 3A
Wnt5a	Wnt family member 5A

12.1 Introduction

According to GLOBOCAN 2018, BCa is the first cancer in incidence and mortality in women (Bray et al. 2018). Even when hereditary factors impact on BCa risk and progression, the nonhereditary are the main drivers of BCa disease (Czene et al. 2002; Willett 2002), some of which are also modifiable factors to be considered for cancer prevention and treatment. Many of these modifiable factors are related to AT content, for example, greater weight, weight gain during adulthood, or body fat distribution (Bray et al. 2018). Thus, obesity, body mass index (BMI), waist-to-hip ratio, central obesity, body fat content, and metabolic syndrome (MeS) influence BCa risk, development, and progression.

Extracellular matrix (ECM) composition has a key role in cancer development and progression and, conversely, it is well known that tumor cells modify ECM to enable their own survival, proliferation, and progression. In addition, ECM components determine the proper adipogenesis and AT integrity. Thus, by modifying ECM, tumor cells alter the surrounding adipocyte homeostasis and induce the transformation of normal adipocytes to CAAs, with low fat and mature adipocyte

markers and overexpression of adipokines, inflammatory cytokines, and high energy metabolites, among other factors (Zhao et al. 2020). Considering AT represents around 56% of breast in nonlactating women (Zhao et al. 2020), this reciprocal interaction between cancer cells and adipocytes becomes more important and understanding the intricated molecular mechanism behind this phenomenon could determine new therapeutic approaches. Also, even in physiological conditions, the breast is a dynamic tissue that suffers several remodeling across women life and where the crosstalk between epithelial cells and adipocytes is fundamental in maintaining their proper development and modifications and conversely a disruption of this dialog could be cause of disease, including BCa (Zwick et al. 2018).

In this chapter, we will discuss the dynamic crosstalk between adipocytes and BCa cells with special emphasis in adipocyte secretions that modify ECM impacting on BCa survival, growth, progression, and resistance to therapy.

12.2 Breast Adipocytes

AT is composed by adipocytes (mature or preadipocytes), endothelial cells, fibroblast, pericytes, stem and progenitor cells, immune cells (macrophages, dendritic cells, mast cells, eosinophils, neutrophils, and lymphocytes), and ECM (Coelho et al. 2013; Miana and Prieto González 2018). According to its location, AT is subdivided in subcutaneous, visceral, intradermic, bone marrow-associated and intramuscular (Ibrahim 2010), been mammary AT an especial subtype of subcutaneous AT.

Adipocytes constitute the major population of AT (Coelho et al. 2013) and deserve special attention since they share a complex and dynamic crosstalk with epithelial cells in mammary gland both in physiological and pathological conditions.

Adipogenesis is a complex process with two master regulators, the nuclear receptor-peroxisome proliferator-activated receptor-γ (PPARγ) and the transcription coactivator CCAAT/enhancer-binding protein α (C/EBPα) (Rosen et al. 2000). Lipid accumulation, adipocyte fatty-acid binding protein 4 (FABP4), and insulin-responsive glucose transporter type 4 (GLUT4) expression determine early stage of differentiation, while adiponectin, leptin, adipose triglyceride lipase, lipoprotein lipase, and perilipin 1 are markers of mature adipocytes (Rybinska et al. 2020). Considering the presence of lipid droplets and mitochondrial content, adipocytes can be divided in white, brown, and beige or brown-like. White AT (WAT) is the most abundant in the body and, in particular, in mammary AT. It has a large lipid droplet and functions as an energy storage but also produce pro-inflammatory molecules and adipokines which gives it endocrine properties in response to inflammatory changes (Kothari et al. 2020). At molecular level, WAT is characterized by leptin and S100 calcium binding protein B (S100B) but not uncoupling protein 1 (UCP-1) expression. Brown AT (BAT) contains small lipid droplets and large spherical and packed iron-enriched mitochondria and contributes to the maintenance of body temperature through mitochondrial UCP-1 protein, which is its molecular marker. Finally, beige adipocytes retain both functions, energy storage and thermogenesis, and express

UCP-1, PPARγ, and leptin and has high mitochondrial content compared to WAT. No matter the subtype, AT is a complex and heterogeneous tissue that could present particular properties according to the specific location or to changes in the microenvironment. Thus, conversion of WAT to beige AT has been reported in response to cold and is called browning (Bartelt and Heeren 2014) and conversely, whitening is the conversion of BAT to beige and has been also observed (Kotzbeck et al. 2018). Also, WAT can transdifferentiate to BAT in response to cold (Himms-Hagen et al. 2000; Peres Valgas da Silva et al. 2019). In physiological conditions, mammary gland epithelium is a plastic tissue that undergoes various remodeling. After birth, mammary gland development and maturation continue during prepuberal to puberal life, and after menarque, respectively. Later in adulthood, remodeling could occur during pregnancy, lactation, and involution (postlactation and age-related). Remarkably, several studies showed that mammary AT interaction with epithelial cells is essential in all these processes (Zwick et al. 2018; Kothari et al. 2020). As well reviewed by Kothari et al., several studies demonstrated that dialogue between mammary adipocytes and epithelial cells is essential for the development of mammary gland (Howlett and Bissell 1993; Zangani et al. 1999). For example, mammary gland development comprises a prepuberal to puberal stage, where ductal branching and terminal end buds take place and, a maturation stage, where alveolar buds develop side branching that occurs upon menarque. It was reported that loss of mammary gland adipocytes causes a diminution of these processes (Landskroner-Eiger et al. 2010).

Pregnancy, lactation, and involution postlactation offer other examples of the dynamic interaction between adipose tissue and epithelial cells in mammary gland and were also well reviewed by Kothari et al. During pregnancy, alveolar development starts and WAT phenotype is modified. Morroni et al. demonstrated that WAT transdifferentiate to pink AT (PAT) with milk secretory capacity (Kothari et al. 2020; Morroni et al. 2004) in a process dependent on the transcription factor secreted phosphoprotein 1 (SPP1) (Prokesch et al. 2014). Also, it has been reported that WAT remains present during pregnancy but with a smaller size (Cinti 2018) but which is the predominant phenomenon is under discussion (Rybinska et al. 2020). Nonetheless, WAT tissue is required for normal alveolar formation and lactation, since it has been reported that deletion of WAT inhibits both processes (Vitali et al. 2012). It has been also demonstrated that BAT transdifferentiate to myoepithelial cells during pregnancy and lactation (L. Li et al. 2017). According to transdifferentiation model, during postlactation involution, PAT must transdifferentiate to WAT, which is dependent on PPARγ (Apostoli et al. 2014). Also, adipocytes with smaller size return to their normal shape by refilling their cytoplasm with lipids (Combs et al. 2004). On the other hand, Secreted Frizzled Related Protein 1 (SFRP1) secretion from adipocytes contributes to remodeling inducing apoptosis of epithelial cells from milk lobules (Clemenceau et al. 2020; Zheng et al. 2017).

Importantly, the aberrant expression of adipocyte markers associated to adipogenesis, breast development, pregnancy, lactation or involution of the mammary gland was also reported in BCa, indicating that AT altered function could be associated to tumor development. For example, it was reported that PPARγ and

C/EBPα are tumor suppressors for BCa development (Apostoli et al. 2014; Lourenço and Coffer 2017; Liu et al. 2019). Also, several transcription factors with functions in adipogenesis, either promoting or inhibiting it, play roles in tumor development and progression (Kothari et al. 2020). There was observed that high expression levels of BAT and beige AT markers in BCa xenografts favor tumor development (Singh et al. 2016).

12.3 Clinical Association Between BCa and Obesity

A clear demonstration that AT has a critical role in BCa development and progression comes from epidemiological studies regarding BCa risk and mortality in patients. Thus, several conditions associated with an increase in AT have an effect in BCa epidemiology (Burton and Foster 1985; Kolb and Zhang 2020; Dibaba et al. 2019) and deserve to be taken into account because they are also usually modifiable risk factors.

Association between overweight (BMI = 25–30 kg/m^2) and obesity (BMI $\geq$ 30 kg/m^2) and BCa was well studied (Burton and Foster 1985; Kolb and Zhang 2020; Cheraghi et al. 2012; Renehan et al. 2008). Thus, several studies demonstrated that BMI is a risk factor for BCa in postmenopausal women (Renehan et al. 2008; Lauby-Secretan et al. 2016). Importantly, a study with more than 87,000 women and another with about 250,000 postmenopausal women have shown that high body weight is associated with increased risk of estrogen receptor positive (ER +) BCa in postmenopausal women (Eliassen et al. 2006; McKenzie et al. 2015). In particular, body weight increasement after menopause raises BCa risk and this association is strong in obese women (Eliassen et al. 2006). When analyzed the role of BMI according cancer subtypes, increased BMI presented stronger association with hormone receptor positive BCa (Yang et al. 2011; Kolb and Zhang 2020; Vrieling et al. 2010).

Further studies should be carried out to determine BMI effect on premenopausal BCa risk. The fact that studies performed in both premenopausal and postmenopausal women show similar results compared to those carried out only in postmenopausal women suggest that overweight is an important factor for both populations (Renehan et al. 2008). However, different results about BMI effect were found in studies performed in premenopausal women. A meta-analysis study showed that BMI is associated with 20% lower risk for ER+ BCa with no association for ER- BCa in premenopausal women (Suzuki et al. 2009). On the other hand, a more recent meta-analysis shows that BMI increases the risk of triple negative BCa (TNBC) in premenopausal women (Pierobon and Frankenfeld 2013). In addition, several studies show that obesity is associated with a BCa worse outcome (Kolb and Zhang 2020). A meta-analyses study demonstrated that BMI increases both all-cause mortality and breast cancer-specific mortality (Chan et al. 2014). Also, obesity constitutes an independent prognostic factor for distant metastases and for death in BCa (Ewertz et al. 2011). A recent meta-analysis including 52,904 patients

demonstrated that BMI increases the lymph node metastasis risk in BCa (Wang et al. 2020). The increase in body weight after diagnosis elevates overall mortality rate (Playdon et al. 2015). Finally, obesity is linked with an increased risk to develop secondary cancer (Druesne-Pecollo et al. 2012).

Considering visceral fat is associated with metabolic disease risk (Neeland et al. 2013) and woman with normal BMI could present visceral fat depots, recent clinical studies also evaluate waist-to-hip ratio. An interesting systematic review showed that while BMI has a neutral or an inverse effect in premenopausal women, central obesity increases their risk of BCa; and for postmenopausal women, obesity is a BCa risk factor but visceral obesity has no effect on BCa risk (Harvie et al. 2003). However, a recent meta-analyses review revealed that waist-to-hip-ratio increases BCa risk and this association is also significant when analyzed only postmenopausal women (Connolly et al. 2002). In concordance with these results, women with a normal BMI but relatively high levels of body fat measured by dual-energy x-ray absorptiometry present increased risk of invasive BCa (Iyengar et al. 2019). Also, elevated waist-to-hip ratio is associated with ER+ BCa mortality in post-menopausal women (Borugian et al. 2003). Finally, it was shown that weight gaining in adulthood elevates BCa risk in women with fattier breast suggesting that a minimum of breast fat is necessary for BCa development and the important role of mammary AT besides visceral fat in BCa (Soguel and Diorio 2016).

Interestingly, a study in 21,000 postmenopausal women investigating obesity in addition with MeS revealed that obesity is a risk factor for BCa but in combination with MeS the risk is even higher (Kabat et al. 2017). MeS is a group of pathophysiological disorders with visceral obesity as a component as comprise at least three of the following characteristics: abdominal obesity (circumference $\geq$ 35 inches in women) (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report 2002), hyperglycemia ($\geq$110 mg/dL), blood triglycerides $\geq$150 mg/dL (in women), HDL-C cholesterol $<$50 mg/dL (in women), and hypertension ($\geq$130/85 mmHg) (Alberti et al. 2009). A systematic meta-analysis study demonstrated that MeS is a risk factor for BCa (Bhandari et al. 2014), being this association even greater in the subpopulation of postmenopausal women (Capasso et al. 2010). Also, evidences indicate that MeS is associated with a high grade of breast tumors (Reeves et al. 2007; Cleveland et al. 2007) and with a higher rate of recurrence and metastasis of BCa (Cao et al. 2015). Importantly, a recent study carried out with data from 94,555 women, demonstrated that MeS is significant associated with BCa mortality risk in postmenopausal women (Dibaba et al. 2019).

Altogether, epidemiological studies suggest that AT tissue; regardless being total, visceral or mammary AT; plays an important role in BCa development and progression.

12.4 CAAs and Crosstalk with BCa

Since adipocytes are the most abundant component of the breast tissue stroma, they make a relevant contribution to cancer progression. As described above, epidemiological evidence has demonstrated the strong relation between obesity and BCa. It is widely known the importance of cancer-adipocyte crosstalk in the modulation of tumor behavior. Consequently, it has been field of extensive study.

Importantly, a few years ago, a new concept has emerged since it was demonstrated that invasive cancer cells can modify adjacent adipocytes, currently called CAAs, which display different characteristics from normal adipocytes. These modified adipocytes, properly located at the invasive front of breast tumors, exhibit fibroblast-like phenotype, smaller size, dispersed small lipid droplets, overexpression of collagen VI, and low expression of adipokines, such as adiponectin, compared to normal adipocytes. Also, CAAs display a different secretome than mature adipocytes: they produce more chemokine (C–C motif) ligand 2 (CCL2) (Fletcher et al. 2017), chemokine (C–C motif) ligand 5 (CCL5) (Cha and Koo 2018), autotaxin (ATX), and pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-1β (IL-1β), interleukin-6 (IL-6) (Bray et al. 2018), tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF), leptin (Pallegar and Christian 2020), etc., all molecules that stimulate invasion and metastasis of BCa. Furthermore, CAAs present reduced expression of the adipocyte differentiation markers PPARγ and C/EBPα as well as their downstream targets FABP4, adiponectin, and hormone-sensitive lipase. The mechanism involved in dedifferentiation of mature adipocytes induced by tumor cells is still unknown, although TNF-α (Mauro et al. 2018), Wnt family member 3A (Wnt3a), Wnt family member 5A (Wnt5a) (Theriau et al. 2017), and stromelysin-3 (MMP11) (Ollberding et al. 2013), all molecules secreted by tumor cells have been related to this process. Interestingly, some authors have proposed that CAAs represent an intermediate form of cancer-associated fibroblast (CAFs) that are the main population of stromal cells in BCa (Bochet et al. 2013).

The interaction between BCa cells and CAAs fosters a pro-oncogenic tumor microenvironment, promoting proliferation, angiogenesis, invasion, and metastasis. Along diverse studies, it has been demonstrated that CAAs establish the communication with BCa cells by releasing soluble factors such as adipokines, impacting in tumor remodeling (Fig. 12.1).

12.4.1 Adipokines Produced by CAAs

As said before, crosstalk between BCa cells and adipocytes is established by soluble factors, and favors proliferation, survival, and metastasis of BCa. The whole set of factors produced by adipocytes, which include more than 600 metabolites,

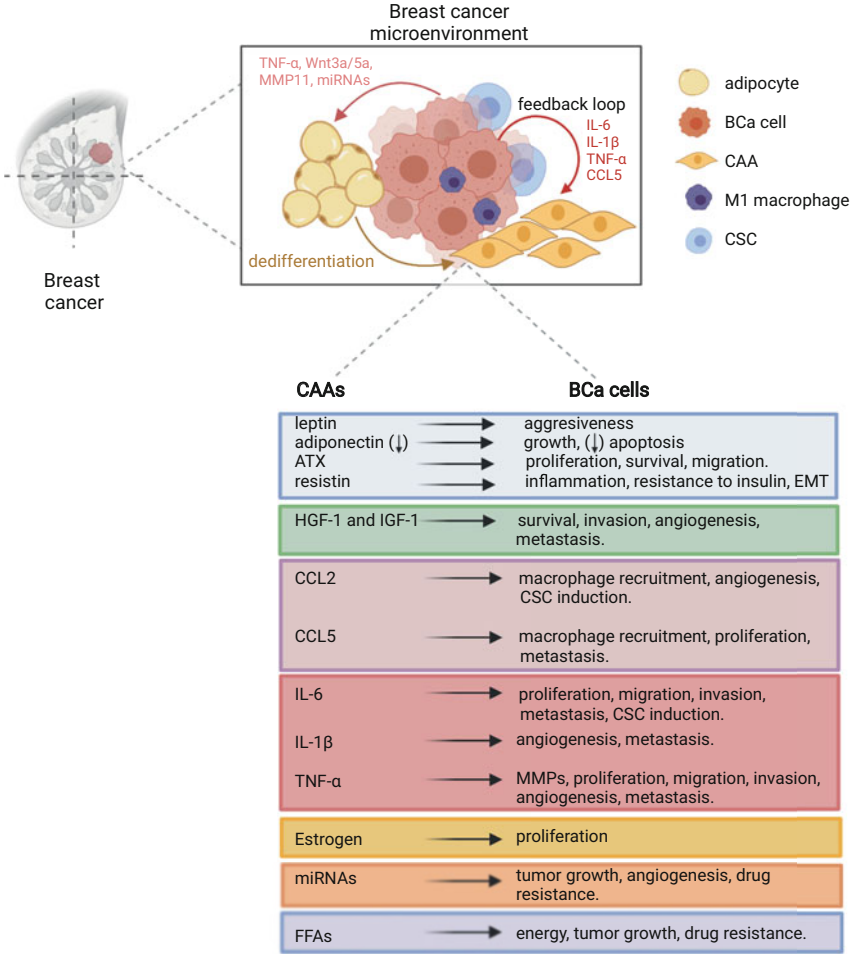

Fig. 12.1 Bidirectional adipocytes–BCa cells crosstalk is fundamental to support tumor growth and progression. BCa cells induce normal adipocytes to dedifferentiate to CAAs, in part through TNF-α, Wnt3a, Wnt5a, MMP11, and miRNAs. In addition, tumor cells induce a metabolic reprogramming of adipocytes in order to ensure high energy molecules for its own benefit. CAAs overexpress adipokines (except adiponectin), inflammatory cytokines, and high energy metabolites, among other factors. High levels of leptin secreted by CAAs promote BCa proliferation, EMT phenotype, progression, and resistance to targeted therapies. Diminished production of adiponectin by CAAs promotes BCa growth, invasion, and suppresses apoptosis. CAAs also secrete ATX (related with proliferation, survival, and migration) and resistin, which induces EMT, BCa cells stemness, and metastasis. Growth factors as IGF, VEGF, HGF, NGF, and PDGF promote survival, angiogenesis, invasion, and metastasis. In particular, HGF induces proliferation, metastasis, and angiogenesis, while IGF-1 induces BCa cells proliferation, EMT phenotype, and stemness. CAAs also produce a battery of inflammatory cytokines: CCL2, that induces BCa cells stemness and neovascularization; CCL5, that promotes invasion and metastasis; IL-6, that impacts on proliferation, EMT, migration, and invasion of BCa cells and is associated with resistance to therapy; IL-1β, associated with invasion, angiogenesis, and metastasis; TNF-α, that induces MMPs and dipeptidylpeptidases and increases BCa cells proliferation, chemoresistance, angiogenesis, and

hormones, and cytokines, are called adipokines. They regulate processes such as fat distribution, insulin secretion, energy consumption, and inflammatory reactions.

12.4.1.1 Leptin

Leptin is a hormone mainly synthesized and secreted by adipocytes. The binding to its receptor ObR induces proliferation, differentiation, inflammation, and nutrient absorption in breast cells (Sánchez-Jiménez et al. 2019). The main role of leptin is to regulate food intake and energy homeostasis leading to fat degradation in adipocytes (Kothari et al. 2020). Leptin circulates in plasma, and its levels increase according to total AT mass (Andò et al. 2014). Moreover, leptin expression has a role in several female cancers, including breast, cervical, endometrial, and ovarian. In particular, in BCa leptin levels in plasma of patients are higher compared to healthy volunteers and constitute a risk factor for BCa development (Niu et al. 2013). Also, leptin levels in plasma from BCa patients correlate with high-grade tumors, poor prognosis, and metastases (Choi et al. 2018). Several studies have shown that leptin promotes BCa growth. In a tumoral context, CAAs produce more leptin than mature adipocytes. Leptin can also be produced by CAFs (Choi et al. 2018). In vitro studies have shown that leptin promotes breast cancer proliferation through diverse pathways. Leptin activates janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol 3-kinase pathway (PI3K)/AKT signaling pathways, promoting the proliferation of BCa cells (Choi et al. 2018). Moreover, leptin accelerates the cell cycle of BCa cells through the expression of cyclin D1 and cyclin-kinase 2 (Chen et al. 2006). It was reported that leptin, in the absence of ligand, can activate ER and human epidermal growth factor receptor 2 (HER2), which impacts in tumor progression and resistance to targeted therapies (Fiorio et al. 2008). Leptin also plays an important role in invasion and metastasis associated with BCa. In BCa cells, it could promote epithelial to mesenchymal transition (EMT) (Wei et al. 2016). Also, leptin produced by mature adipocytes in coculture with BCa cells together with IL-6 promotes local invasion and eventually metastasis. Adipocyte-derived leptin also drives self-renewal of BCa stem cells and chemoresistance in mouse breast tumors (Wang et al. 2018).

Additionally, leptin together with IL-1 favors angiogenesis by the induction of VEGF/VEGFR expression. It also upregulates IL-1-/IL-1R signaling, contributing to the angiogenic process (Zhou et al. 2011). On the other hand, leptin produced by

Fig. 12.1 (continued) metastasis. CAAs also exhibit augmented expression of aromatase, thus increasing estrogens production, which is highly relevant in BCa growth. Adipocytes suffer lipolysis driven by BCa cells. FFAs from adipocytes are transferred to BCa cells, where they can be used for newly synthesized membrane phospholipids from tumor cells. Also, miRNAs secreted by CAAs could be important in tumor growth, angiogenesis, and drug resistance Finally, BCa cells secrete inflammatory cytokines that induce CAAs and immune cells to secrete pro-inflammatory molecules in a cyclic feedback loop

adipocytes enables BCa progression by regulating immune cells as well. For example, IL-8 is produced by tumor-associated macrophages under leptin stimulus (Li et al. 2016). Further, leptin could weaken CD8+ T cell effector functions through activating STAT3- fatty acid β-oxidation and inhibiting glycolysis thus impairing breast tumor progression (Zhang et al. 2020).

12.4.1.2 Adiponectin

It is suggested that adiponectin works in the opposite way to leptin in the context of BCa progression, since it suppresses growth and invasion and induces apoptosis of BCa cells (Dieudonne et al. 2006; Körner et al. 2007) through protein kinase AMP-activated catalytic subunit alpha 1 (AMPK) activation (Dieudonne et al. 2006) and PI3K/AKT inhibition. It was reported that adiponectin secretion is reduced in CAAs, and is considered a protective factor against tumor progression (Wu et al. 2019). Several studies suggest that it is the leptin adiponectin ratio which determines the BCa risk and progression, and not leptin or adiponectin levels separately. Thus, high leptin to adiponectin ratio correlates with increased risk of postmenopausal BCa (Ollberding et al. 2013) and TNBC progression (Sultana et al. 2017).

12.4.1.3 Autotaxin and Resistin

ATX is a secreted glycoprotein produced by adipocytes but also platelets, endothelial cells, fibroblast, and cancer cells. Its function consists in the conversion of lysophosphatidylcholine to lipid signaling molecule lysophosphatidic acid mediating cells renewal, proliferation, survival, and migration (Lee et al. 2018). Besides, inflammation produced by tumor BCa cells triggers an increase of ATX expression in CAAs and fibroblasts, generating a feedback loop that favors tumor progression. ATX may be part of the connection between BCa and obesity since is augmented in subcutaneous AT and blood of obese patients (Ferry et al. 2003; Boucher et al. 2005).

Resistin is another adipokine observed in inflammatory zones. It can also be secreted by monocytes, macrophages, and bone marrow cells (McTernan et al. 2002; Patel et al. 2003). Among resistin functions, it is known that it increases inflammation, resistance to insulin and atherosclerosis as increases low-density lipoproteins (LDL) (Schwartz and Lazar 2011). It can also induce EMT and stemness in BCa, thus promoting the metastatic potential of BCa cells (Avtanski et al. 2019). As other adipokines circulating in blood, it can act systemically and locally. In fact, in circulation of obese patients is found in high levels (Makki et al. 2013). Interestingly, in BCa tissues, it can also be produced by CAAs, and its receptor adenylyl cyclase-associated protein 1 (CAP1) is expressed in primary human tumors. Moreover, its high expression in BCa tissue of patients was associated with aggressiveness and poor prognosis (Rosendahl et al. 2018).

12.4.1.4 Growth Factors

AT also secretes many growth factors that play known functions in several cancer, such as insulin growth factor (IGF), VEGF, hepatocyte growth factor (HGF), nerve growth factor (NGF), and platelet-derived growth factor (PDGF) (Peeraully et al. 2004; Pallua et al. 2009). In particular, they promote BCa survival, angiogenesis, invasion, and metastasis. These factors have also been studied in the majority of tumors, including the different stages of BCa progression. In addition, the increase of these factors in BCa milieu leads to resistance to therapies (Meyer et al. 2013; Voudouri et al. 2015; Linklater et al. 2016).

HGF and IGF-1

Both growth factors can be secreted by adipocytes or preadipocytes, also contributing with BCa progression.

HGF binds to its receptor, c-Met, located at tumor cells, and signals for proliferation, metastasis, and angiogenesis (Gallego et al. 2003). Interestingly, HGF was found to be higher both in adipocytes and serum from obese patients compared to lean individuals (Bell et al. 2006). Furthermore, c-Met expression was observed in the invasive front of BCa, which is located in direct contact with CAAs (Edakuni et al. 2001).

Insulin-like growth factor 1 (IGF-1), through binding to IGF-1R expressed in several subtypes of BCa (Law et al. 2008), activates PI3K/AKT and MAPK pathways, which impact in their proliferation. Importantly, insulin resistance, associated with obesity and other metabolic diseases, causes an increase in both insulin and IGF-1 levels, which constitute a risk factor for BCa (Esposito et al. 2013; Key et al. 2010). Moreover, IGF-1 was found to be twofold increased in CAAs from obese patients compared to lean individuals (D'Esposito et al. 2012; Nam et al. 1997). IGF-1R was described to have a role in cancer stem cells (CSCs) development and maintenance, EMT process and regulation of tumor microenvironment (Seccareccia and Brodt 2012). In addition, in hormone-dependent BCa, IGF-1R is coexpressed with ER-α, thus the cobinding of their ligands induce proliferation of tumor cells (Surmacz 2000).

12.4.1.5 Inflammatory Factors

It is well known that patients suffering from obesity and cancer display chronic inflammation and high levels of C-reactive protein (Dossus et al. 2014). Particularly in obesity, local inflammation is histologically recognized by the presence of "crown-like-structures," composed by dead/dying adipocytes surrounded by macrophages. These macrophages, which acquire an M1 pro-inflammatory phenotype (Engin 2017), are recruited in response to fat accumulation with the subsequent

increase in chemokines CCL2 and CCL5 (Chen et al. 2005; Keophiphath et al. 2010). Free fatty acids (FFAs) and TNF-α signaling in macrophages and adipocytes, respectively, trigger the transcription of pro-inflammatory genes such as prostaglandin E2, TNF-α, IL-1, and IL-6, generating a feedback loop at the AT from breast and visceral fat of obese women (Rybinska et al. 2020).

CCL2 and CCL5 Chemokines

CCL2 is also known as monocyte chemoattractant protein-1 (MCP-1). It can be produced and secreted to extracellular environment by different cells composing the tumor microenvironment, such as tumor cells, fibroblasts, endothelial cells, and infiltrating monocytes (Melgarejo et al. 2009). CCL2 binds to the G-protein-coupled receptor C–C motif chemokine receptors 2 and 4 (CCR2 and CCR4), and is a chemoattractant for immune cells, especially monocytes, to the inflammatory site (Melgarejo et al. 2009). CCL2 also induces the activity of CSCs, by the induction of Notch receptor 1 (NOTCH1) expression (Tsuyada et al. 2012), and is significantly associated with neovascularization (Ueno and Zhang 2011). Noteworthy, it was suggested by Arendt et al. that crown-like structures, associated with malignant progression of BCa, are formed as consequence of macrophages recruitment by mammary epithelial cells-secreted CCL2 (Arendt et al. 2013).

CCL5 (previously called RANTES) can be produced by different types of cells in physiological processes. Its expression can also be found in BCa tissue at high levels, and can be produced by mesenchymal stem cells, as well (Khalid et al. 2015). CCL5, by binding to its receptor CCR5, induces invasion and metastasis of BCa (Velasco-Velázquez et al. 2012; Sax et al. 2016). This axis was found to be highly activated specifically in TNBC and HER2+ BCa. In fact, CCL5 expression was statistically associated with poor disease-free survival and overall cancer survival in patients with early HER2-positive BCa (Zazo et al. 2020), and the abundance of CCL5 in peritumoral AT of TNBC patients is also correlated with metastasis and poor overall survival (Song et al. 2018).

It is hypothesized that CCL5 is secreted by stromal cells into the tumor milieu and is recognized by CCR5, activating AKT/mTOR pathway, thus promoting tumor metastasis (Kim et al. 2018). On the other hand, CCL5 contributes to the inflammatory microenvironment since it could recruit macrophages and other immune cells (Keophiphath et al. 2010). Interestingly, a study showed that the invasiveness of MDA-MB-231 cells induced by human adipocytes was reduced by antagonizing CCL5 with specific peptides and antibodies (D'Esposito et al. 2016). Then, it can be suggested that antagonizing CCL2 and/or CCL5 in peritumoral adipocytes might be a potential target for BCa and its metastasis.

IL-6, IL-1(β) and TNF-α

IL-6 is a pleiotropic cytokine involved in various biological processes, such as immune regulation, hematopoiesis, and tumorigenesis. It can be secreted by adipocytes and acts locally and systemically. Locally, it suppresses lipogenesis and secretion of adiponectin (Bachelot et al. 2003). Under pathological conditions like obesity and cancer, the level of IL-6 secreted by adipocytes is significantly higher. IL-6 produced by adipocytes and BCa cells has been reported to impact on proliferation, migration, and invasion of BCa cells by modulating the expression of EMT-regulating genes (Gyamfi et al. 2019). Also, IL-6 increases aromatase expression by adipocytes and BCa cells, as well as estrogen synthesis, thus contributing with BCa progression (Purohit et al. 1995). It was demonstrated in vitro that upon the interaction of adipocytes and TNBC cells, the increment of IL-6 expression drove to higher migration and aggressiveness of these cancer cells (Nickel et al. 2018). Additionally, in clinical specimens from HER2-positive BCa, it was demonstrated that IL-6 induced the production and maintenance of breast CSCs through nuclear factor kappa-B (NF-κB) and STAT3 signaling pathways, then promoting BCa progression and resistance to therapy (Liu et al. 2018). Moreover, it is known that high levels of IL-6 in serum are correlated with poor prognosis of BCa (Rybinska et al. 2020).

IL-1β is involved in different steps of BCa progression. It is secreted into the extracellular microenvironment mainly by blood monocytes and tissue macrophages (Shen et al. 2017). It was observed that when adipocytes are cocultured with cancer cells, there is an increase in the expression of pro-inflammatory factors including IL-1β (Dirat et al. 2011). Importantly, this cytokine has been identified as a potential predictor of bone metastasis in BCa patients, since it increases the expression of osteoprotegerin, a promoter of invasion and metastasis of BCa. IL-1β can also promote tumor angiogenesis, by inducing the expression of VEGF and its receptors on endothelial cells (Tulotta and Ottewell 2018).

In addition, IL-1 has an important role since it is mainly secreted by AT in the tumor milieu. Moreover, IL-1 expression is increased in BCa, and is considered as poor prognosis, since this cytokine induces a secondary response by activating the secretion of other pro-inflammatory molecules and also by altering the expression of adhesion molecules strongly involved in metastasis. For these reasons, IL-1 is considered the most alarming factor in BCa evolution (Perrier et al. 2009).

TNF-α plays a physiological role in premenopausal non-obese women by regulating adipogenesis and lipid storage in adipocytes, thereby the total volume of AT (Cawthorn and Sethi 2008). TNF-α plays important functions in the tumor microenvironment, as well. It can be secreted by tumor and stromal cells, macrophages, or adipocytes. This cytokine increases tumorigenesis mainly by induction of matrix metalloproteases (MMPs) and dipeptidylpeptidases (Wolczyk et al. 2016). Noteworthy, in the serum of healthy women, TNF-α is generally not detected, while it is elevated in BCa patients (Alfano et al. 2017). TNF-α participates in BCa development, proliferation, chemoresistance, angiogenesis, and metastasis (Balkwill 2009).

In addition, it exerts a regulatory effect of estrogen synthesis (Liu et al. 2016) and enhances lipolysis in human adipocytes (Zhang et al. 2002).

12.4.1.6 Estrogens

Estrogens result from the aromatization of dihydrotestosterone that is catalyzed by aromatase. As well known, mammary epithelium is sensitive to estrogens and almost all BCa respond to this hormone either by canonical or noncanonical pathways acting in BCa cells or even by estrogens effect in the stromal cells (Péqueux et al. 2012). According to aromatase expression, the main sources of estrogens production are the ovaries in premenopausal women, but after menopause, AT becomes the principal site of its synthesis. Moreover, conditions of high AT, as obesity or high BMI, are linked to higher estrogen levels in circulation (Gérard and Brown 2018; Brown et al. 2017), hence they constitute one of the classical molecular explanations for increased risk of BCa in this subgroup of the population, and this issue has been deeply reviewed (Gérard and Brown 2018). Molecular mechanisms have been studied to explain the increased production of estrogens in AT of obese patients and have support on epidemiological studies (Gérard and Brown 2018). Thus, obesity induces overexpression of inflammatory factors such as TNF-α, IL-6 or CCL2, produced by adipocytes. Furthermore, other pro-inflammatory factors secreted by immune cells are also associated with increased expression of aromatase and, in consequence, of estrogens level (Purohit et al. 2002; Gérard and Brown 2018). Consistent with this, both aromatase expression and activity were found to be increased in AT of obese patients (Morris et al. 2011; Purohit et al. 2002; Lorincz and Sukumar 2006). However, in contrast, several studies have shown that circulating estrogens have a protective role against the development of breast cancer in obese women (Suba 2013; Key et al. 2003). Interestingly, it was demonstrated that in breast AT, the inflammatory factors associated with obesity induce a shift in the aromatase promoter PI.4 to the promoter PI.3/I, which is more sensitive to inflammatory factors, leading to an increase in estrogens level (To et al. 2015; Gérard and Brown 2018). Some authors suggested that estrogens produced locally in mammary AT could have more impact on BCa growth and progression associated with obesity, as estrogens levels are higher in the tumor microenvironment compared to circulation (van Landeghem et al. 1985; Rybinska et al. 2020).

12.4.1.7 microRNAs in AT and BCa Cells Crosstalk

MiRNAs are noncoding RNAs of 21–25 nucleotides considered global regulators of gene expression that play roles in multiple biological processes contributing to cellular homeostasis. Dysregulation of miRNA expression is associated with numerous pathologies, including cancer and metabolic disorders (Rottiers and Näär 2012; Takahashi et al. 2015). MiRNAs have functions in all steps of BCa development and progression (Kaboli et al. 2015). Also, it was demonstrated that they are master

regulators of metabolism involved in processes such as cholesterol secretion, synthesis and degradation, fatty acid synthesis, lipoprotein degradation (Fernández-Hernando et al. 2013), maintenance of insulin, glucose and liver lipid homeostasis, and b-cell differentiation in pancreatic islets (Rottiers and Näär 2012). Several studies reported aberrant expression of circulating miRNAs in obesity and MeS (Deiuliis 2016; Karolina et al. 2012; Ramzan et al. 2020). These observations support the idea that miRNAs could also be part of AT and BCa crosstalk. In fact, several studies suggest that miRNAs could be part of the signal necessary for the transformation of normal adipocytes into CAAs and, conversely, miRNAs secreted by CAAs could be important in tumor growth, angiogenesis, and drug resistance (Bandini et al. 2019; Wu et al. 2019). A recent study showed that in vitro coculture of BCa cells with mature adipocytes induces proliferation, migration, and invasion and differential expression of 98 miRNAs in BCa cells where miR-3184-5p was the most increased while miR-181c-3p represent the most decreased miRNA whose direct targets were Forkhead box P4 (FOXP4) and PPARα, respectively (Rajarajan et al. 2019). Also, it was reported that miRNA-144, miRNA-126, and miRNA-155 secretion by BCa cells induce adipocytes to CAAs transformation (Sun et al. 2019). This study revealed that exosomal miR-144 induces beige/brown differentiation of adipocytes, and exosomal miRNA-126 is involved in metabolic reprogramming of adipocytes (Sun et al. 2019). Another study showed that exosomal miRNA-155 induces adipocyte lipolysis promoting aggressive phenotype of cancer cells (Wu et al. 2018). MiRNA-105 secreted by BCa cells induces metabolic reprogramming of CAAs and CAFs ensuring high energy conditions to support tumor cells growth (Yan et al. 2018). Interestingly, obesity downregulates p16INK4A protein in breast adipocytes increasing leptin secretion in a mechanism mediated by miR-141 and miR-146b-5p which is associated with EMT in breast ductal epithelial cells (Al-Khalaf et al. 2017). As showed above, several studies demonstrate a role of miRNAs secreted by tumor cells in adipocyte phenotype and transformation but further studies are needed to unveil the effect of miRNAs secreted by adipocytes in BCa growth and progression.

12.5 Metabolic Reprogramming

It is hypothesized that tumor cells, upon interaction with adipocytes, can induce a reprogramming of adipocytes metabolism in order to adapt it for tumor benefit. Since adipocytes are considered tremendous energy storage that provides high-energy metabolites (Reeves et al. 2007), their reprogramming could provide the tumors a high potential to proliferate. This reprogramming involves the metabolic regulation of almost all macronutrients, such as carbohydrates, lipids, and aminoacids (Choi et al. 2018).

It was observed in coculture assays with BCa cells and adipocytes that the latter suffer lipolysis driven by tumor cells. Besides, in this experiment, fatty acids (FAs) from adipocytes were transferred to BCa cells (Balaban et al. 2017) and this event

favored BCa cells growth by increasing mitochondrial β-oxidation. This fact could explain the smaller size and less lipid droplets in CAAs (Dirat et al. 2011; Andarawewa et al. 2005). In turn, these transferred FAs can be used as substrates for newly synthesized membrane phospholipids from tumor cells (Menendez and Lupu 2007).

Importantly, FFAs secreted by CAAs can be used for the biosynthesis of lipid-signaling molecules that act as tumor promoters (Kotzbeck et al. 2018).

Interestingly, obese adipocytes provide more FAs to cancer cells than nonobese adipocytes (Balaban et al. 2017). Moreover, cell surface fatty acid translocase (CD36) is detected in most breast cancer tissues located adjacent to AT, and is recognized as a marker of cells initiating metastasis, even in other tumors (Pascual et al. 2017).

Adipocytes instructed by tumor cells rearrange their metabolism to glycolysis, thus releasing energy-rich metabolites such as lactate and pyruvate. Monocarboxylate transporters (MCT) also play an important role in the transport of these metabolites to BCa cells. In particular, MCT1 and MCT4 are overexpressed in BCa tissue (Baenke et al. 2015; Johnson et al. 2017). In vitro coculture experiments revealed that tumor cells induce MCT4 expression in adipocytes facilitating lactic acid efflux (Sun et al. 2019). A clinical study demonstrated that MCT1 and MCT4 expression in BCa tissue correlates with poor prognosis of patients. Moreover, MCT1 correlation with poor prognosis is stronger when is linked to MCT4 expression in adjacent AT. In addition, ER–BCa cells express high levels of MCT1 compared to ER+ tumors (Li et al. 2018).

Adipocytes that undergo glycolysis also produce ketone bodies, which serve as source for ATP production in invasive cancer cells. These ketone bodies induce mitochondrial biogenesis in BCa cells, increasing their ability to growth (Martinez-Outschoorn et al. 2012). Moreover, induction of ketone-specific gene signature was shown to be associated with worse outcomes in BCa patients (Argilés et al. 2014).

12.6 Therapeutic Approaches Focused on BCa Cells and AT-Crosstalk

There are a huge variety of approaches to treat the different subtypes of BCa. In this section, we will focus especially in those related to molecules differentially expressed in the AT under tight interaction with BCa tissue.

While inhibition of adipogenesis is considered as option, since a misbalance in AT volume fuels BCa progress, this strategy is not a first option since adipogenesis is a physiological process, and its inhibition could lead to adverse effects. Also, CAAs present a dedifferentiated phenotype, hence inhibition of adipogenesis could have controversial effects.

Adipokines and cytokines secreted by adipocytes are a more suggested target for therapy. Specifically, leptin depletion or treatment with leptin receptor antagonists is

frequently employed in BCa treatment. Also, inhibition of ATX-LPA axis from AT is the focus of the latest strategies (Marshall et al. 2012).

Another option consists in reinforcing the effect of beneficial adipokines, as is the case of adiponectin. Peptide-based adiponectin receptor agonists, such as ADP-355, inhibit BCa growth in vitro and in xenografts (Otvos et al. 2011). In addition, (−) catechin flavonoid increases adiponectin expression (Si et al. 2007).

Blockade of inflammatory factors produced by CAAs is also a possible strategy. Some compounds are already employed in the clinical practice or are involved in clinical or preclinical studies, such as Tocilizumab (monoclonal antibody anti-IL-6R), Canakinumab (monoclonal antibody anti-IL-1β), Infliximab (monoclonal antibody anti- TNF-α), and AZD3695 (inhibitor of MCT1) (Polanski et al. 2014). In addition, it has been shown that inhibitors of CCR5 (receptor for the chemokine CCL5) Maraviroc and Vicriviroc can reduce infiltration and invasion of BCa cells (Levy 2009).

It is also possible targeting the metabolic interactions between BCa cells and adipocytes. An example of this case is Myricetin, which suppresses the accumulation of lipid droplets in the adipocytes (Chang et al. 2012). Suppression of FA oxidation constitutes a potential target, as FA is source of energy for BCa cells once transferred from adipocytes. Trimetazidine, which is an inhibitor of FA oxidation, induces apoptosis in cancer cells (Andela et al. 2005). Finally, another approach consists in inhibiting the transport of FA. BMS 309403, an inhibitor of FABP4, is a candidate assayed in preclinical studies (Nieman et al. 2011).

12.7 AT and Resistance in BCa Therapy

Secretome from adipocytes and cellular interactions not only can fuel BCa growth but also can promote resistance to multiple therapies for BCa, including chemotherapy, hormonal therapy, radiotherapy, and immunotherapy. Several studies have reported the negative influence of the adipose microenvironment both in resistance to chemo- and hormonal therapies, such as doxorubicin, tamoxifen, and fulvestrant, and a few examples will be shown in this section.

Major vault protein (MVP) is a transport-associated protein overexpressed in BCa cells as a result of interaction with adipocytes. In a coculture assay with adipocytes and BCa cells treated with doxorubicin, adipocytes induced an MVP-related multidrug-resistant phenotype, which could be relevant in obesity-related chemoresistance (Lehuédé et al. 2019). Another mechanism involving AT promoting resistance to therapy is the stimulation of CSCs, mediated through adipocyte-derived leptin (Delort et al. 2019). Besides, it was observed that leptin impaired the effect of hormonal tamoxifen therapy (Delort et al. 2019), and interestingly in overall and tamoxifen treated BCa patients, leptin expression correlated with poor prognosis (Chen et al. 2013).

These data reflect that adipokines could predict drug resistance to BCa therapy, evidencing the relevance of exploring more exhaustively the crosstalk between BCa cells and adipocytes.

12.8 Concluding Remarks

Mammary tissue constitutes a structure with high plasticity where the tight interaction between epithelial cells and AT is determinant in both physiological conditions and disease. Adipocytes comprise a key component of AT and strongly contribute to tumor development, growth and progression. Under unhealthy conditions such as obesity, adipocytes contribute to creation of a pro-inflammatory microenvironment that could impact on BCa development. Moreover, upon BCa development, these lipid-rich cells provide an important source of energy for the neighboring tumor cells, which establish a tight communication with adipocytes. This crosstalk promotes dedifferentiation of adipocytes into CAAs, which in turn produce several pro-tumoral stimuli, generating a cyclic feedback-loop.

The cooperation between adipocytes and BCa is emphasized in many epidemiologic studies, showing a clear connection between obesity and BCa outcome. For this reason, in the last years, AT became a challenging target for BCa therapies. Moreover, AT is also involved in resistance to radio-, chemo-, and immunotherapy. Thus, a deep comprehension about the crosstalk between BCa and adipocytes is under the focus of several studies. This knowledge would provide a very valuable tool for developing novel therapies in order to tackle BCa.

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Chapter 13
Extracellular Matrix as a Metabolic Niche in Cancer

Anna Sebestyén, Titanilla Dankó, Dániel Sztankovics, Dorottya Moldvai, Ildikó Krencz, Regina Raffay, and Gábor Petővári

Abstract Heterogeneity in tumor mass with an altered tumor microenvironment is a hallmark of cancer metabolism, which provides a metabolic niche for cells. Tumor cells with their higher metabolic plasticity have competitive advantage in tumor tissue evolution. The high glucose consumption, in parallel with impaired vascularization related to tumor growth, usually results in lactate production, extracellular glutamine depletion, and increasing acidification. These alterations completely rewire the activity of non-tumor cells (cancer-associated fibroblasts, inflammatory cells, adipocytes, endothelial cells, etc.). Additionally, amino acids, lipids, or other structural matrix elements and their degradation products—as nutrients—have critical importance in building the biomass for cancer growth. Changes in the extracellular matrix influence and regulate metabolite composition of this niche; moreover, these modifications can induce metabolic alterations in tumor cells (e.g., Warburg effect, OXPHOS phenotype, and autophagy), alter metabolic crosstalk between tumor and non-tumor cells, generate metabolic heterogeneity in tumor tissue, and finally affect the homeostasis of the organ-body, as well. This metabolic symbiosis can initiate and/or promote the aggressiveness and progression of cancer.

13.1 Metabolic Rewiring and Altered Cellular Bioenergetics

Growing cancer has many well-known hallmarks—including metabolic rewiring and altered cellular bioenergetics which are highly influenced by the special environment, nutrients, and growth factors—and requires anabolic properties to facilitate cell growth. During the adaptation to the permanently changing conditions in tumor tissues, cells need to rearrange the activity of metabolic pathways (catabolic/anabolic

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processes) to achieve nutrient supplements, bioenergetic forces regarding tumor cell growth and survival. This feature—metabolic rewiring—has an important function in tumor development and progression. Stromal components can support or inhibit tumor cell proliferation, survival, and adaptation (Hanahan and Weinberg 2011). Accordingly, the complex stromal microenvironment with its matrix elements and the non-tumorous cells deserves special interest (Schwörer et al. 2019). The altered metabolic by-products of tumor cells influence the functions and even the extracellular matrix (ECM) composition of the surrounding cells, and also respond to the tumor cell-derived paracrine factors. Oncogenic alterations in both tumorous and parental cells disrupt normal organ and tissue ECM structure (Egeblad et al. 2010; Li et al. 2018; Elia and Haigis 2021). These alterations are able to reprogram non-malignant cells, and influence their produced ECM proteins and other metabolic by-products (as potential nutrients). These drive the supporting functions of ECM to force tumor cell adaptation and cancer progression.

Proliferating cancer cells reorganize the tissue architecture and the adjacent stroma; furthermore, tumor growth becomes dependent on vascularization and stromal supports. Several studies suggest that tumor/stroma ratio, and the structure of the stroma (e.g., compact, desmoplastic stroma) may correlate with the prognosis of cancers (e.g., colon and breast cancers) subtype dependently (Kramer et al. 2019; van Pelt et al. 2018). However, to use tumor/stroma ratio as a reproducible prognostic factor needs robust clinical validation in future prospective studies. The special roles of certain cell types—e.g., cancer-associated fibroblasts (CAFs) and immune regulatory cells—were described in the progression and formation of several tumors in the last three decades (Hanahan and Coussens 2012; Casazza et al. 2014; Louault et al. 2020; Karta et al. 2021). Alterations in ECM—including matrix proteins, metabolites, and acidification—are intensively studied in parallel with the non-tumorous stromal cells (mesenchymal, immune, and endothelial cells) in the microenvironment (Fig. 13.1). It was proposed that *heterogeneity of metabolic pathway alterations in tumor mass with altered tumor microenvironment is a hallmark in cancer* metabolism, which provides a metabolic niche in cancer (Elia and Haigis 2021; Pavlova and Thompson 2016). It was highlighted that ECM nutrients produced by several different cells provide building blocks for tumor cell growth. In addition, the accumulation of metabolic "waste"/by-products has several regulatory functions that influence the behavior of surrounding cells and the remodeling in ECM (Baltazar et al. 2020). The secreted metabolic products (as lactate or different amino acids) by certain cells can be utilized in a competition among various cell types (metabolic symbiosis) where tumor cells can orchestrate the consumption and gain several advantages. Metabolites have both self-mediated paracrine effects in the microenvironment and/or induced actions via signaling mechanisms in stromal cells or the related matrix elements. These effects could have special roles both in the matrix and in the cellular elements of tumor tissues. In this chapter, we summarize the roles of ECM in metabolic symbiosis and review how these affect tumor microenvironment and growth in a complex manner (Yoshida 2021).

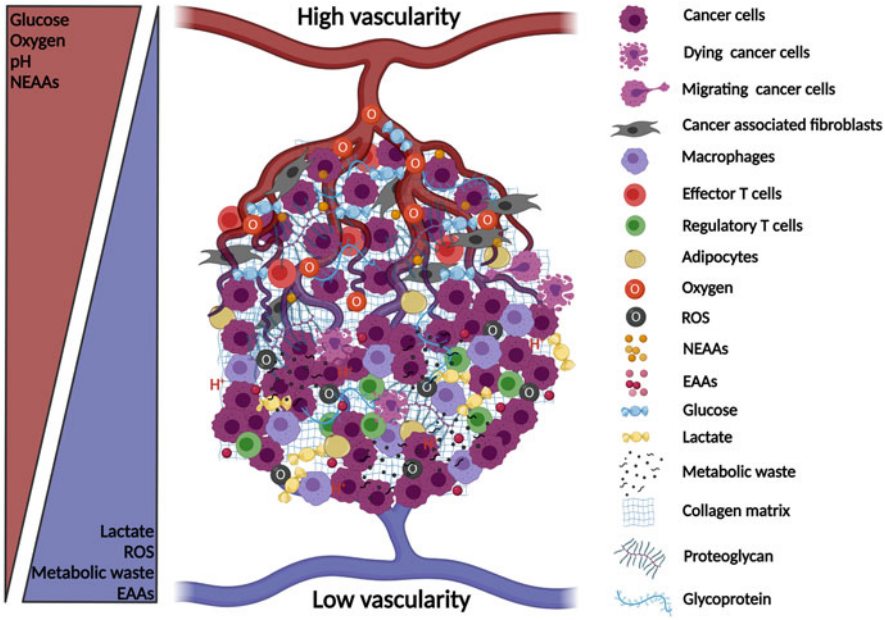

Fig. 13.1 Altering metabolic compartments including metabolic microenvironment in tumor tissue. Several elements of the microenvironment and their altered metabolic activity contribute to tissue and metabolic complexity. Interactions/communications and the symbiosis of different cell populations include the altered concentration of macromolecules and metabolites. Altering nutrients, amino acids (essential and non-essential amino acids—EAAs/NEAAs), glucose, lactate, waste, and the developing pH gradients, oxygen concentration differences, reactive oxygen pressures (ROS—reactive oxygen species), and ECM element deposition rewire the tissue microenvironment and increase cellular and metabolic heterogeneity of the tumor tissue in correlation with vascularization. Gradients, altering conditions were labeled in blue/red triangles on the left side of the figure. Cellular elements (proliferating, surviving, and dying cancer cells, effector and regulator T cells, macrophages, adipocytes, and fibroblast—cancer-associated fibroblasts) and extracellular proteins, proteoglycans, and metabolites were also indicated

13.2 Extracellular Matrix Elements Compensate Starving Conditions During Tumor Cell Growth and Survival in Tissue Microenvironment

Tumor growth and survival need both bioenergetic supply and building blocks for new macromolecule biosynthesis at starving conditions. These processes can be maintained by the uptake of ECM-derived metabolites. Glucose and amino acid consumption are the main sources of carbon required for proliferating cell mass in tumor tissues. *Glucose consumption* is high in correlation with the glycolytic activity of cancer cells; however, there are other metabolic pathways that require glucose (including pentose phosphate pathway—generating pentose phosphates for nucleotide synthesis and NADPH, hexosamine pathway—requiring for protein

glycosylation, gluconeogenesis—generating glycogen in glucose storage, amino acid synthesis, and one-carbon metabolism—producing NADPH, purines, glutathione, methylation, etc.). Compared with normal cells, the glucose influx and glycolysis are accelerated in tumor cells due to the overexpression of transporters and glycolytic enzymes. It is also well known, a general statement that "anaerobe glycolysis is enhanced and OXPHOS capacity is reduced in many cancer cells". The well-known Warburg effect, the lactate producing anaerobe glycolysis, is the more suitable and fast way to gain energy for cancer growth, but it yields less ATP/glucose. Therefore, growing new cells need other metabolic intermediates for maintaining macromolecule biosynthesis, as well. These demands can be supplied by truncated TCA cycle and other TCA fulfilling processes. These mechanisms highly reduce the concentrations of glucose and some additional metabolites (including glutamine or other amino acids, lipids) in the ECM.

Glutamine utilization and TCA cycle fulfilling mechanisms, the anaplerosis, support tumor growth/survival in hypoxic-pseudohypoxic conditions (Wise and Thompson 2010). Additionally, glutamine uptake facilitates the neutralization of reactive oxygen species (ROS) and nucleotide biosynthesis. As glutathione plays a role in the exchange of non-essential amino acids (NEAAs) such as glutamine, cysteine, and serine, glutamine and glutamate levels can be reduced in the ECM. Consequently, the ammonia level is elevated due to amino acid consumption intra- and extracellularly. Moreover, the inappropriate vascularization, the constant delay in nutrient and oxygen supply—further decreases the level of building block metabolites in the ECM (Lane et al. 2020; Tímár et al. 2021). Acetate, as acetyl-CoA source, could also be used to support lipid/fatty acid synthesis by ACSS2 (acetyl coenzyme synthetase) overexpression or Ras-transformed cells scavenge unsaturated fatty acids from phospholipids in several cancers (Lovinfosse et al. 2016; Huang et al. 2018; Currie et al. 2013). These alterations also support the growth and survival of tumor cells; however, the non-malignant cells have no such adaptation possibility or plasticity in this nutrient- and O_2-depleted microenvironment. Thus, tumor cells with their higher metabolic plasticity have competitive advantages in selection and tissue evolution. The most important metabolic pathways and some key elements in nutrient utilization regarding cellular metabolic adaptation were summarized in Fig. 13.2.

Additionally, growing tumors invade the microenvironment, cause injury, and inflammation as permanent stress, which damage cellular and matrix components. These result in degradation-derived supplements, where ECM elements and matrix proteins provide nutrient sources for tumor cells in case of poor vascularization (Johnson et al. 2016). The matrix could compensate starving conditions at protein, amino acid, and lipid metabolism levels (Commisso et al. 2013; Olivares et al. 2017; Guillaume et al. 2019). The affected and actively participating stroma of the developing tumor has many features similar to wound healing which influence tumor cell growth and survival (Dvorak 1986, 2015; Singer and Clark 1999; Balkwill and Mantovani 2001). Cytokine and other factors producing tumor cells alter the cellular and metabolite compositions. These changes are also accompanied by the accumulation and/or decrease of different metabolites and alter the non-tumorous cell

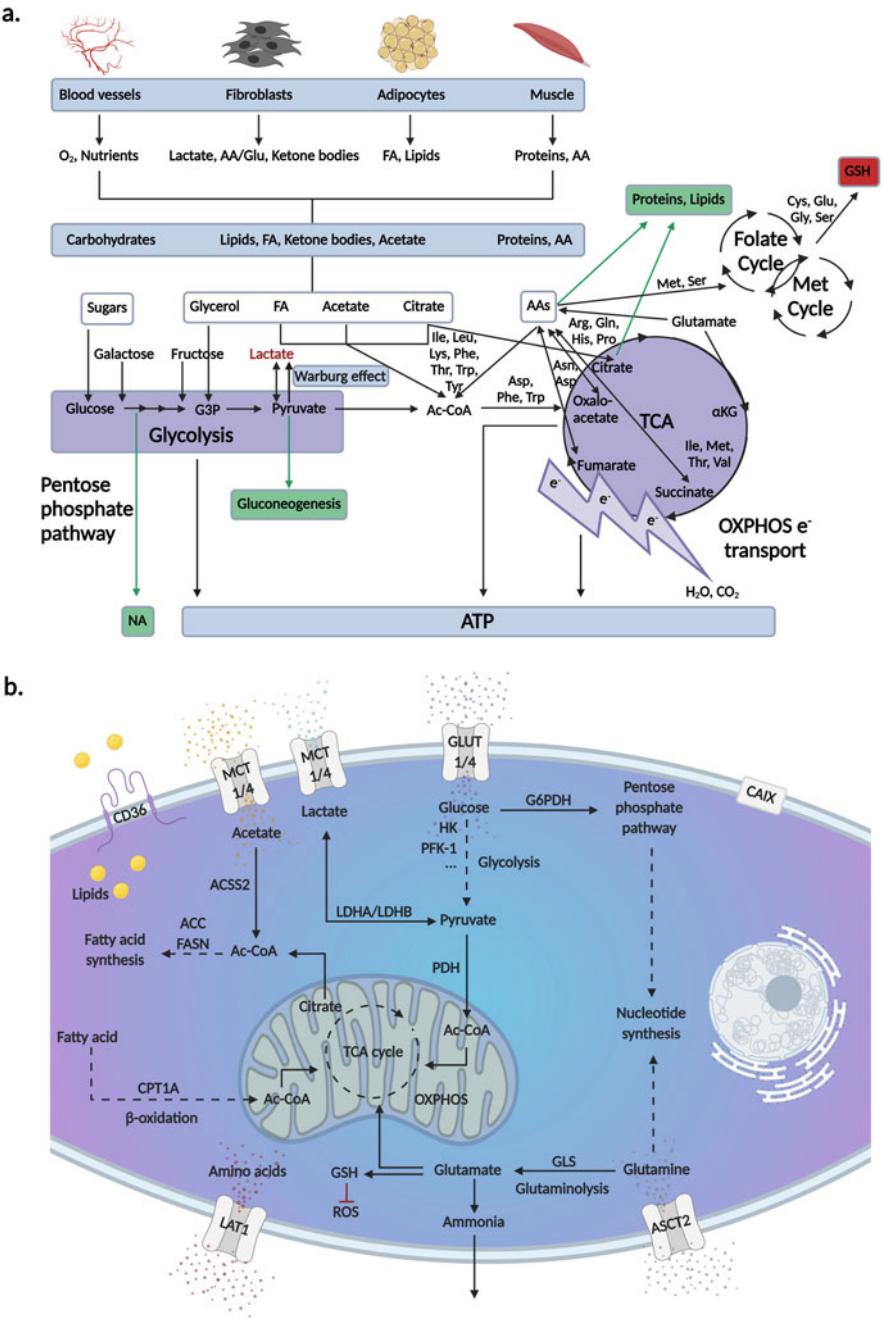

Fig. 13.2 Metabolic pathways in nutrients and building block cycles. (**a**) Cancer cells can undergo metabolic changes to adapt to altering microenvironments. Metabolic plasticity helps to use available metabolites which come from different sources (vessels, fibroblasts, adipocytes, muscle, or other cellular elements). Metabolic compartments O_2, nutrients, lactate, ketone bodies, lipids, proteins, and amino acids (AA) allow supply flexibility by using multiple metabolites for growing and surviving tumor cell requirements. Carbohydrates (e.g., sugars), lipids, fatty acids (FA), or

composition in the microenvironment. Tumor cells facilitate further reorganization of the surrounding cellular milieu, including immune, mesenchymal cells, fibroblasts, and other cells with non-tumor origin (e.g., adipocytes). Stromal cells participate in the coordination of tissue regeneration; therefore, these produce several regulatory factors (cytokines, growth factors) to adjust tissue remodeling (Hynes and Naba 2012). Monocytes release many small molecules, as danger-associated-molecular patterns, which are usually paracrine metabolites, nucleotides, ATP, pro- and/or anti-inflammatory cytokines. The increased amount of immunosuppressive regulatory T (T_{reg}) cells, M2 macrophages, and myofibroblasts contributes to the production of tissue regenerating cytokines (TGFβ; PDGF) and chemotactic factors initiating ECM remodeling. The produced cytokines and other factors promote the trans-differentiation of myofibroblasts to *cancer-associated fibroblasts (CAFs)*. CAFs are characteristic for many solid cancers, and support the proliferation, adaptation, and survival mechanisms as well as facilitate the metastatic capacity of tumor cells. Cancer cell metabolism itself has a direct effect on the establishment of tumor-promoting microenvironment. In addition with tumor growth-related high nutrient consumption, the nutrient pool of tumor tissues could be completely exhausted, as a consequence of metabolic waste release into the microenvironment (Guillaume et al. 2019).

In 1986, *Dvorak* described cancer as a "wound that does not heal". This statement was confirmed by several observations since many characteristics of growing tumors are similar to healing wounds: such as *high rate of glucose consumption, lactate production, and extracellular glutamine depletion* after trauma and hypoxia (Dvorak 1986, 2015). Based on these, the metabolic alterations occurring in tumor tissues resemble the wound healing processes (Byun et al. 2018; Dvorak 1986).

Fig. 13.2 (continued) other metabolites can fuel the metabolic pathways (glycolysis, pentose phosphate pathway, TCA cycle, and ATP generation). Moreover, several nodes in these pathways (their metabolites) present different building blocks for the newly produced cancer cells to synthetize their macromolecule requirements (green arrows, nucleic acid—NA, protein, and lipid synthetic processes). This metabolic plasticity and flexibility contribute to the production of certain important building blocks (green background) and energy for growing tumor cells (gray-blue background) and maintaining redox homeostasis (red background, glutathione—GSH) help to survive starved, injured situations, e.g., in dormant state (glycerol 3 phosphate—G3P; αKG—alpha-ketoglutarate; Ac-CoA—acetyl-coenzyme A; amino acids were indicated using their common three-letter abbreviations). (**b**) Schematic figure of the above summarized main pathways in the cell which help to orientate the associations of several metabolic pathways during metabolic rewiring. The figure does not contain all metabolic enzymes and processes but we tried to present the ones which were referred in the main text (ACSS2—acetyl coenzyme synthetase, ACC—acetyl coenzyme A carboxylase, Ac-CoA—acetyl coenzyme A, LDHA/B—lactate dehydrogenase A/B, HK—hexokinase, PFK-1—phospho-fructokinase-1, G6PDH—glucose-6-phosphate dehydrogenase, PDH—pyruvate dehydrogenase, OXPHOS—oxidative phosphorylation, CPT1A—carnitine palmitoyltransferase 1A, GSH—glutathione, GLS—glutaminase, CAIX—carbonic anhydrase IX, GLUT1/4—glucose transporter 1/4, MCT1/4—monocarboxylate transporter 1/4, LAT1—L-type amino acid transporters, ASCT2—alanine, serine, cysteine transporter 2)

13.3 The Effects of Lowering Glucose Concentration and High Lactate Level (Tissue Acidification)

Tumor cell proliferation is supported by several oncogenic mutations and hyperactivated growth signals. These induce the expression of certain genes to serve bioenergetic demands. Increase in the transcription of myc-regulated glucose transporters and glycolytic enzymes speeds up glucose uptake and catabolism. Since glucose concentration has several regulatory functions in ECM homeostasis, the reduced glucose level—as an important metabolic checkpoint—exerts numerous effects in the tumorous ECM. The high glucose consumption, in parallel with impaired vascularization, results in about *90% decrease of glucose concentration* in interstitial tissue fluids during intensive tumor growth (Gullino et al. 1964). Simultaneously, the immune response activating cells, inflammatory T cells, macrophages, and fibroblasts are mainly glycolytic and require glucose for their ATP consumption. High ^{18}F-flourodeoxiglucose (FDG) uptake of the tumor and other non-tumor cells (as CAFs or activated immune cells) in the cancer microenvironment is a well-known problem of routine analyses in PET-CT (e.g., the FDG positivity of immune cells). This phenomenon is associated with both the Warburg phenotype of rewired non-tumor cells and the metabolic symbiosis (Sugita et al. 2021).

The increased aerobe and/or anaerobe (pseudohypoxia) glycolysis (Warburg effect) were described in tumor cells by *Otto Warburg* about 100 years ago (Warburg et al. 1927). The elevated intracellular level of lactate initiates the lactate/H^+ transport accomplished by the monocarboxylate transporters (MCTs) which increase *tissue acidosis*. The consequences of acidic microenvironment and its tumor-promoting effects were clarified in the last decade (Payen et al. 2020). O_2 consumption and the resulted CO_2 release in tumor mass also contribute to the decrease of the pH induced by the HCO_3^- transports of carbonic anhydrases (CAs). CAIX, together with other bicarbonate transporters, promote the extracellular H^+ accumulation and acidification (Benej et al. 2020; Gillies 2021; Lee and Griffiths 2020). CAIX inhibitors, which are already studied in clinical trials, may help to inhibit the metabolic adaptation in the future (McDonald et al. 2020; Jamali et al. 2015; Aldera and Govender 2021; Lau et al. 2017).

13.3.1 Immunosuppressive Effects of Increased Lactate Level in the Extracellular Matrix

Effector T cells have high glucose demand in immune defense, and the lowering glucose impairs many immune effector functions in the microenvironment such as IFNγ production and T helper 1 differentiation. In addition, the depleted glucose shifts T_{reg} differentiation and function. T_{reg} cells are less sensitive to lowered glucose concentration than other effector T cells. In T_{reg} cells, the lowered glycose

concentration and as a consequence the decreasing ATP level elevate AMPK activity and reduce glucose oxidation. In parallel, however, lipid/fatty acid oxidation can be increased to compensate glucose depletion in T_{reg} cells. Based on these, the available glucose concentration could be an important metabolic checkpoint to regulate anti-tumor immune response of T cells in tumor mass (Siska et al. 2020; Angelin et al. 2017). It was described that PD-1/PD-L1 interactions and their signs in disrupting CD28-mediated co-stimulation inhibit glucose transport and consumption in activated T cells. Regarding this, it was detected that PDL1 immune checkpoint therapy upregulates GLUT1 expression of effector T-cell population to uptake glucose more effectively in competition with tumor cells in their microenvironment. The regulatory role and the potential competition for available glucose in the microenvironment were confirmed by other findings since adaptive T-cell therapy is less effective in highly glycolytic tumors (Chang et al. 2015). There are many publications about glucose demand and Warburg phenotype of *inflammatory M1 macrophages*, as well. All these underline that enhancing glycolytic flux promotes M1 macrophage differentiation and low glucose concentration reverses this and promotes the polarization of anti-inflammatory M2 macrophage in the microenvironment (Bader et al. 2020). Tumor-promoting capabilities are characteristic for M2 macrophages involving immunosuppressive and angiogenic effects, as well as stromal remodeling.

It was summarized that increasing lactate level inhibits the functions of NK and NKT cells during cancer progression. It was documented that lactate induces the apoptosis of NK cells (Harmon et al. 2019) and the silenced lactate dehydrogenase (LDH) caused the better cytolytic activity of NK cells in a pancreatic cancer xenograft model (Husain et al. 2013). In correlation with these, a recently published work described that lactate could inhibit mTOR signaling, block IFNγ, and IL-4 in NKT cells (Xie et al. 2016).

Increasing lactate can influence cytokine production of immune regulatory cells, which can prevent the final differentiation of dendritic cells (remaining tolerogenic) leading to an increase in immunosuppressive IL-10 production (Nasi et al. 2013). In addition, lactate promotes the overexpression of IL-23 in different types of tumor cells (e.g., melanoma, colon, breast, and gastric cancers) (Langowski et al. 2006), which enhances the expression of IL-17, matrix metalloprotease 9 (MMP-9), induces angiogenesis, and reduces the number of cytotoxic T cells, as immunosuppressive effects (Shime et al. 2008).

The above-described examples are evidences which indicate that lactate helps to avoid the recognition of tumor cells and promotes tumor progression through its diverse immunosuppressive effects in tissue microenvironment.

13.3.2 The Effects of Lactate in Metabolic Symbiosis (Including Several Functions of Fibroblasts and Endothelial Cells)

Tumorigenic alterations lead to PI3K/Akt/mTOR pathway hyperactivation in tumor cells and increase glucose uptake as metabolic driving forces. The lowered oxygen

level, the hypoxic conditions, or the pseudohypoxic regulatory changes induce the Warburg effect and the accumulation of lactate in the microenvironment (de la Cruz-López et al. 2019; Koppenol et al. 2011). However, in oxygenated environment both tumor and surrounding other cells (mainly fibroblasts) can uptake and catabolize lactate and other metabolic intermediates (e.g., carbohydrates, ribose, certain lipids, fatty acids, acetate, and amino acids) which ensure the requirements of their growth and survival. These situations can contribute to varied metabolite concentrations in the extracellular environment and alter metabolic milieu (Thompson and Bielska 2019). Furthermore, tumor cells have special adaptation processes and advantages in nutrient- and O_2-depleted ECM supported by the rearrangement of catabolic and anabolic processes, and force CAFs to initiate nutrient production and release for themselves (as nutrient supplies for cancer progression). Local metabolic pathway redistributions of nutrient utilization and building block cycles as well as cellular *metabolic symbiosis* serve tumor cell adaptation.

Decreasing glucose concentration resulting from tumoral consumption lowers intracellular ATP level in starving stromal fibroblasts. This activates AMPK, consequently inactivates mTOR kinase, and switches on autophagy for bioenergetic survival mechanism in fibroblasts. These processes and the autophagy-dependent mechanisms guide amino acids into the ECM to replenish the consumed nutrients and selectively promote tumor cell growth (Sousa et al. 2016). Further effects of low glucose in the growing tumors create a *circulus vitiosus* in tissue vascularization. Low glucose level decreases phospho-fructokinase-1 (PFK-1), and consequently the proliferation and migration of sprouting *endothelial cells*. This impaired vascularization initiates the decrease of nutrient and glucose supply in certain parts of the tumor mass (Cantelmo et al. 2016). All these effects of lowered glucose level in the ECM promote the transformation of tumor-associated stromal myofibroblasts to CAFs, and alter the distribution of immune cells participating in the matrix remodeling of growing tumor mass. The above-described situation with intensive aerobe glycolysis and hypovascularization causes *lactate accumulation* in the ECM (García-Cañaveras et al. 2019). Additionally, the high intratumoral and vascular concentrations of lactate increase the risk of metastasis and death.

Tissue/tumor heterogeneity and the adaptation to oxygen gradient (related to different vascularization) lead to rational energy consumption which is in correlation with lactate transport. This promotes the so-called *"waste" (e.g., lactate) use in oxygen-rich microenvironment* near the vessels. For directing this shuttle, MCTs help the lactate flux. MCT1 is involved in lactate influx and efflux in all cells. In addition, MCT4 expression is characteristic for tumor cells in hypo-oxygenated microenvironment, where effective lactate efflux is necessary (in highly glycolytic cells). Recent studies emphasize the importance of *both glucose and lactate*—as metabolic substrates. These two metabolites can be nutrients for cells depending on their microenvironment and oxygen supply (Payen et al. 2020).

This implies that *lactate is not a real waste* in the ECM of tumor mass. Lactate can be taken up and oxidized in cells with mainly oxidative phosphorylation (OXPHOS) metabolism or in chemotherapy-resistant cells (Taddei et al. 2020). For example, in spheroid-forming breast cancer cells (mammospheres), lactate

oxidation can promote long-term survival and late tumor initiation (Lamb et al. 2015). Several new results described that lactate among other alternative nutrient substrates can be oxidized in quiescent "stem"-like cancer cells with OXPHOS phenotype (Farnie et al. 2015; Ózsvári et al. 2020; Intlekofer and Finley 2019). It was also suggested that the therapy-resistant cells are also able to use lactate in the course of tumor evolution (Ippolito et al. 2016). Regarding these, the inhibition of lactate uptake and/or oxidation can disturb the survival and adaptation of cancer cells in disease progression, especially in surviving "dormant" cells (Akkoc et al. 2021).

Lactate released from hypo-oxygenated cells can be taken up either by benign or malignant highly oxygenated cells in the so-called "two- or three-compartment models" in different cancers (breast, pancreas, lung cancer, etc.). This *metabolic symbiosis* can promote or initiate the aggressiveness of cancer and additionally, the altered metabolite levels could have several indirect tumor growth-promoting effects both on malignant and on non-malignant cells. Based on these, *lactate is an important oncometabolite* in this symbiosis and its consequence—tissue acidosis—has further extracellular and intracellular signaling effects. These effects influence the secretion of growth factors both by the tumor cells and by other cellular elements of the tumor stroma, including *fibroblasts, endothelial cells, and adipocytes*. CAFs start to secrete HGF, ILs, other cytokines, and growth factors (TGFβ, IFNs, FGFs, VEGF, etc.) and in addition, release regulatory proteins, metabolites, and/or epigenetic regulators (e.g., regulatory miRs) including exosomal transport (Gorchs and Kaipe 2021). These microenvironmental rewirings promote the motility, migratory, and invasive alterations of tumor cells.

Intracellularly lactate can function as a direct inducer of hypoxia response in hypoxia-related protein stabilization. Lactate can bind and stabilize oxygen level-regulated NDGR3 protein (this prevents its PHD2/VHL-dependent degradation) (Lee et al. 2015). Therefore, NDRG3 protein is able to bind c-Raf, activate Raf-Erk pathway, and induce NF-kB, which promotes, for example, VEGF and bFGF production in cancer cells and growth factor receptor expression in the surrounding endothelial cells tumor type dependently (Sonveaux et al. 2012).

13.3.3 Direct Receptor-Ligand Signaling Effects of Lactate on Tumor Cells

It was described that several G-protein-coupled receptors can be activated by metabolites responding to secreted products of metabolism (fatty acids, mono- and disaccharides, amino acids, or metabolic intermediates ketone bodies, lactate, succinate, etc.). In the extracellular niche, the direct receptor-ligand effect works through lactate-activated G-protein-coupled receptor GPR81, also known as hydroxycarboxylic acid receptor 1 (HCAR1) (Parks et al. 2020). This receptor can be found in the plasma membrane and other intracellular membrane organelles; its activation lowers cAMP level and inhibits PKA signaling mechanisms. The receptor

function could be regulated by direct lactate receptor interaction or conformation modification in acidic microenvironment. Recent publications reported the elevated expression of GPR81 in many cancers (e.g., breast, cervical, and liver) and cancer cell lines (e.g., colon, breast, lung, and pancreatic cancer cell lines). These alterations showed association with the aggressiveness, therapy resistance, and survival of cancer cells in model systems.

Furthermore, proton-sensitive lactate sensors (GPR4/65/68/132) were also described which are influenced by acidic tissue microenvironment (low pH) in macrophages (Parks et al. 2020). The H^+-sensing mechanisms can activate intracellular Ca^{2+}, cAMP, ROS, and these alterations lead to CREB-mediated alterations in different cells through cellular signaling network including MAPK pathways and protein kinase activations. In addition, these further increase matrix MMP expressions and epithelial-mesenchymal transitions (Parks and Pouysségur 2017) influencing tissue remodeling and cancer progression.

13.3.4 Lactate Induces Tissue Remodeling

High lactate concentration and its consequence—acidosis—in the microenvironment (the acidic niche) reorganize tissue elements which stimulate cancer cell overgrowth and invasion into normal tissues (Gillies and Gatenby 2015). Acidic microenvironment is linked to proteolysis of ECM elements, as a result of the synthesis and the activation of MMPs. These proteins can derive from tumor and stromal cells; moreover, macrophages and fibroblasts redistribute their lysosomes to the cell periphery in acidic microenvironment. Besides, cathepsinB is secreted in that way directly into the microenvironment. *Kobayashi* suggested first that this membrane cathepsinB-enhanced proteolytic network could have a role in metastatic matrix remodeling (Kobayashi et al. 1993; Sameni et al. 1995; Glunde et al. 2003).

Hypoxia itself and its consequences—e.g., the lactate production and the acidic microenvironment—could activate certain factors as latent TGFβ and increase the expression and/or activity of other lysosomal degradation enzymes. These alter the architecture of ECM, degrade collagens (e.g., collagen IV), increase enolase-1, and plasminogen activation (Taddei et al. 2013). Finally, the unbalanced ECM degradation, the remodeling of basement membrane, and stiffness contribute to the cancer cell invasion, as well. These alterations could help matrix remodeling which assist progression, alter the polarity and adhesion capacity of the tumor cells, and additionally, propagate invasion and metastasis. For example, collagen deposits are elevating in the early progression of breast cancer which increase tissue stiffness with their cellular consequences (Najafi et al. 2019). These result in the expression changes of lysyl oxidase and hydroxylases catalyzing intramolecular crosslinks between collagens and elastins (Qi and Xu 2018). Both enzymes are overexpressed and could hydroxylate collagens in response to hypoxic conditions. The elasticity and strength of the matrix, the increase/modification of crosslinking the matrix elements were described in many cancers, these usually negatively influenced the

progression (e.g., breast, head and neck, pancreatic, bronchial cancers glioblastomas, and melanomas) (Deville and Cordes 2019). Additionally, non-enzymatic crosslinking by glycanation/transglutamination and fibronectin-mediated collagen reorganization could also occur in the later stages of tumor progression.

Based on the previously summarized alterations in the exchange of glucose and lactate of the tumor microenvironment, the increasing amount of lactate in the ECM contributes to the progression and therapy resistance of different cancers (Fig. 13.3). These in situ alterations favor migration and metastasis with the help of altered matrix-specific intracellular molecule distribution. Therefore, quantification of lactate and pH alterations has an increased interest in cancer research (Lau and Heiden 2020).

13.4 Other Altering Non-cellular Elements as Metabolic Factors in the Extracellular Matrix

During nutrient, glucose, and/or amino acid deprivations, the uptake and the catabolic degradation of ECM elements can also help to maintain bioenergetic homeostasis (Muranen et al. 2017; Olivares et al. 2017). In this situation, non-tumorous cells increase MAPK phosphorylation, decrease mTOR activity, and induce autophagy to cover the energy demand for survival. In this situation, CAFs could be forced by tumor cells to produce metabolites as nutrients through autophagy activation. Based on several studies, both nutrient deprivation and oxidative stress activate autophagy and stabilize HIF1α in CAFs and stromal fibroblast in cancers (Linares et al. 2017; Martinez-Outschoorn et al. 2010; Valencia et al. 2014; Ono et al. 2009).

Amino acids, as nutrients, and even their uptake from the extracellular environment have critical importance in cell growth for building the biomass of proliferating tumor cells (Hosios et al. 2016). These processes deplete the amount of NEAAs (Eagle 1955) in the ECM. As it was previously mentioned, glutamine is highly consumed by tumor cells promoting intensive cell growth in several ways, e.g., it can fulfill TCA cycle intermediates (anaplerosis). Additionally, glutamine—similarly to serine—is important for nucleotide biosynthesis. Glutamine, cysteine, and glycine have a role in maintaining redox homeostasis through glutathione exchange and/or precursors for glutathione production (Valencia et al. 2014). Tumorous and normal cells are in competition for amino acids in nutrient-depleted conditions (fast lowering amino acid level). At starving situation, the oncogene-driven increased expression of transporters and certain other mechanisms, as macropinocytosis of ECM proteins, can give some advantages for fast proliferating tumor mass (Su et al. 2021). Nutrients are used in a well-organized and optimized symbiosis in a heterocellular microenvironment. In a cooperative metabolic way, all cells consume, survive, and additionally, tumor cells mainly proliferate. In metabolic stress forced by the environment, CAFs can utilize glutamate and lactate; furthermore, they provide cysteine

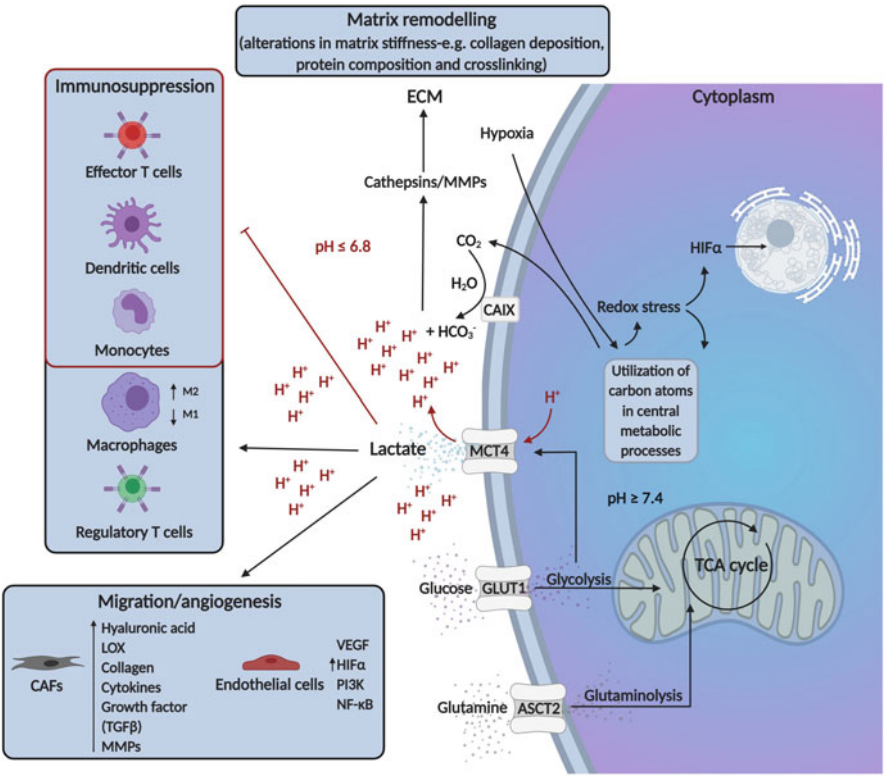

Fig. 13.3 Altering lactate concentration and pH, the role of acidic microenvironment in tumor mass. Both tumor and stromal cells can produce lactate related to their oxygen supply and/or tumorigenic alterations (e.g., pseudohypoxia). The accumulation of lactate and its consequence, the acidic pH, are associated with increased aggressiveness, which inhibit anti-tumor immune response and induce resistance to therapy, invasion, and metastasis. Lactate can be used as an alternative nutrient supply for well-oxygenated tumor cells; negatively regulates innate and adaptive tumor-infiltrating immune cells (e.g., impairs monocyte differentiation, decreases antigen presentation; inhibits immune effector cells, natural killer cells—NK and cytotoxic T cells; promotes immunosuppressive cells—M2 macrophage differentiation and myeloid-derived suppressor cells, and regulatory T cells), these contribute to immune escape mechanisms. The expression profile of metabolite transporters (CAIX—carbonic anhydrase IX, GLUT1—glucose transporter 1, MCT4—monocarboxylate transporter 4, ASCT2—alanine, serine, cysteine transporter 2) can also change at the surface of tumor cells to facilitate uptake and release of several nutrients from the ECM to serve and give selective growing source for the tumor cell proliferation. The tumor cells and the recruited cancer-associated fibroblasts (CAFs) start to produce cytokines, growth factors, and metabolites for the growing demand of tumor cells. Additionally, the acidic microenvironment activates several matrix-embedded enzymes (e.g., matrix metalloproteases—MMPs, cathepsins) for matrix degradation and remodeling, liberating tumor cells from tissues and promoting migration and metastasis. Based on these changes, the cancer-generated lactic acidic microenvironment through reprogrammed metabolism represents a critical immunosuppressive and tumor-promoting niche. Lactate is rather a metabolite than a "waste" product in these situations

and glutamine for cancer cells. Moreover, this adaptation mechanism—especially cysteine and glutamine uptake and their use in cancer cells—can be inhibited by effector T cells (Yang et al. 2016; Sousa et al. 2016). In other cases, the forced autophagy in CAFs provides alanine and several dipeptides for tumor cells, as well. Besides, fatty acids and adipocytes can provide glutamine-arginine conversion from citrulline for growing tumor cells. These feeding effects may support cancer cell proliferation in tumor mass, e.g., in correlation with obesity (Incio et al. 2016; Tajan and Vousden 2016; Meyer et al. 2016).

It was described that many amino acids (glutamine, arginine, cysteine, serine, tryptophane) are required for effective T-cell response in the microenvironment, as well. Similar to acidification, the high glutamine uptake of tumor cells and the lowering glutamine concentration in the ECM suppress the glutamine utilization of T cells and reduce their proliferation (Nakaya et al. 2014). Macrophages and endothelial cells are also very sensitive to depleted amino acid situations. The lowered amount of available cysteine and serine, which are essential for NADPH and glutathione, induces ROS to accumulate at a toxic level. In addition, endothelial cells depend on NEAA-supported TCA cycle anaplerosis, and some other VEGF-mediated angiogenic proliferations which could also be affected in nutrient-deprived ECM.

The three-dimensional matrix of extracellular molecules, the ECM, gives not only the structure but the biochemical, bioenergetic source for the growing tumor mass. In tumor progression and evolution, the components and the formation of this matrix alter in correlation with the consequence of hypoxia and acidosis. The major components—*collagens, fibronectin, laminin, elastin, and proteoglycans* can be degraded by low pH-activated MMPs, and other proteases. Moreover, this remodeling and the acidic microenvironment activate endoglycosidase to digest heparan sulfate glycosaminoglycans (HS-GAG) (Hammond et al. 2014). In addition, other released molecules such as lipoprotein- and exosome-delivered substances can influence the functions of proteoglycans (PGs) (Fuster and Esko 2005). The acidosis alters ligand-receptor interactions among cancer cells and ECM through the induced proteolytic activity, heparinase, tumor-promoting ligand, growth factor, cytokine, and chemokine, as well as lipoprotein productions and exosome secretions. Furthermore, the negatively charged GAGs attract protons in the ECM locally, and at the membrane surfaces (EV or cell membranes). The metabolite release-influenced pH alterations can elaborate matrix and several proteoglycan-bounded cytokines to circulation to help the re-localization of these factors to distant sites and form pre-metastatic niche for migrated, metastatic cells. Tumorigenic and hypoxic alterations (as glycolytic drivers) can be influenced by hyaluronan-mediated degradation (hyaluronidase) in correlation with metabolic rewiring of cancer cells. Additionally, hyaluronidase promotes glycolysis by receptor tyrosine kinase-mediated way which is required for the elevation of cell migration in tumorigenesis (e.g., in breast cancer models) (Sullivan et al. 2018).

Proteoglycans (PGs) are proteins which are conjugated with glycosaminoglycan polysaccharides. Localized either at intra- or extracellular site, or as co-receptors on the cell surface, PGs avidly bind basic proteins and other positively charged

molecules as a consequence of their highly anionic character. The structures and the diverse functions of these molecules influence both tumor development and progression (Fuster and Esko 2005; Iozzo and Schaefer 2015). In addition, the negative charge and presence of PGs in the matrix can influence proton (H^+) distribution and alter local pH (Maroudas et al. 1988). The GAG chains and the related charge-mediated bindings have further dynamic alterations in the matrix composition and tissue homeostasis. Cytokines and growth factors can bind to these molecules and stored or released depending on the actual pH. Moreover, several activated proteolytic enzymes (metalloproteases, heparanase) can liberate these factors and alter the activity of growth factor/cytokine-receptor signaling network both in malignant and in non-malignant cells. It is also suggested that cell membrane-bounded HS proteoglycans can interact with lipoproteins, low-density lipoproteins (LDLs) and efficiently help in lipoprotein internalizations as receptors, and even as co-receptors in classical LDL and/or lipoprotein receptor-mediated pathways (Menard et al. 2018; Christianson and Belting 2014). In acidic microenvironment, the alterations in these functions have to be investigated further.

In addition, lipid droplet (LD) accumulation is general in the acidic ECM of chronic inflammation (which usually also occurs in tumor growth) (Shyu Jr. et al. 2018). This condition significantly facilitates the PG-mediated lipid uptake (Oörni and Kovanen 2006). It was reported that increased lipoprotein uptake assisted by surface heparan sulfate proteoglycans (HSPGs), and the followed cellular storage of LD are in correlation with increased survival and metastatic capacity of hypoxic glioblastomas and osteosarcoma cells (Menard et al. 2016; Cortini et al. 2021). Regarding cellular metabolism, new functions were recently described related to some PGs in the ECM. New structural functions have been described in correlation with biglycan and decorin. Biglycan can be synthesized de novo and released from the ECM during several stress responses including proteolytic matrix degradation. Upon this, it can act as Toll-like receptor ligand (as a potent danger signal) and induces inflammatory reactions by inflammatory cytokine secretions (IL1β, TNFα, and chemokines) (Schaefer et al. 2017). Additionally, biglycans can stabilize and bind HIF2α to influence erythropoiesis and stimulate NOX enzymes, ROS, and VEGF which are contributing to inflammation-induced genetic alterations and genomic instability. Decorin also affects the cellular milieu influencing secretion and the release of several angiogenic and growth factors. Moreover, the newly developed function of this PG is to influence metabolic activity of certain cells. Decorin, as an agonist of VEGFR2, facilitates Peg3-dependent accumulation of autophagic vacuoles in endothelial cells, which means that this ECM-localized PG can regulate cellular autophagy (Buraschi et al. 2019). It was also published that decorin accumulation is developing during nutrient deprivation in parallel with autophagy and mitophagy induction. This regulatory function of an ECM PG was underlined in in vivo KO experiments, where the autophagy flux and the maturation of autophagosomes were impaired in Dcn−/− mice (Gubbiotti et al. 2015).

Based on these, decorin as an ECM-localized PG can regulate cellular autophagy in metabolic processes (Buraschi et al. 2019) (Gubbiotti et al. 2015).

It is also known that *exogenous lipids, fatty acids, lipid vesicles, and endogenous lipids* produced by cells using fatty acid synthase activity are stored in LDs (Corbet et al. 2016). LD accumulation was shown in a number of different cancers (Bensaad et al. 2014; Cotte et al. 2018), and it was also suggested that this phenotype may promote chemoresistance, as well. In case cholesteryl ester accumulation is targeted, the migratory capacity of several highly metastatic tumor cells (e.g., pancreas cancer or glioma) can be reduced (Li et al. 2016; Bemlih et al. 2010). In triple-negative breast cancers, however, the LD accumulation showed no correlation with metastatic potential; the accumulated LDs could have a prognostic role in metastatic properties tumor type dependently (Wright et al. 2017). Hypoxia and the related acidosis activate LIPIN-dependent mechanisms, with SREBP-mediated lipogenic programs, and induce lipoprotein internalizations enhancing Erk signaling (Chen et al. 2018). It was also proposed that fatty acid oxidation and synthesis are mainly balanced in healthy tissues, whereas cells decrease acetyl CoA carboxylase (which prevents lipid production from fatty acid oxidation) during adaptation in acidic microenvironment. Usually, glutamine utilization and reductive glutamine metabolism could balance lipid metabolism with de novo lipogenesis (Metallo et al. 2011). These processes are very important and promoted by active mitochondrial functions and TCA cycle. The exogenous lipids were also considered to influence metastasis based on some new publications; CD36 and/or FABP4 are hypoxia-induced by the extracellular lipid receptors at the cell surface. Using these mechanisms, cholesterol and fatty acid uptake can participate in progression and initiate tumor spreading (Ladanyi et al. 2018; Gharpure et al. 2018). As it was previously mentioned, obesity influences both normal and tumorous ECM and their homeostasis, as well. In correlation with these effects, the prevalence and the risk of several cancers can be increased. Obesity-related endocrine, inflammatory, and metabolic factors assist in tumorigenesis, which can alter nutrient-dependent metabolic regulation and cellular signaling network such as IGF and PI3K/Akt/mTOR axis activity, respectively. Obesity-associated cytokines (e.g., IL-6 and TNFα) and adipokines (e.g., adiponectin and leptin) increase estrogen synthesis (e.g., in breast cancers) and promote tumor growth (Rajesh and Sarkar 2021). These factors, their release, and local accumulation in the tumor matrix are very important metabolic players of the microenvironment.

Inorganic polyphosphates (polyP, ATP/ADP)—linear orthophosphate residues with high energy bonds of phosphoanhydrides—are able to form and function to store biochemically useful energy both intra- and extracellularly. Phosphotransfer for energy-consuming also needs to be considered in ECM; alkaline phosphatases and adenylate kinases function both in cells and in ECM and are also crucial in ATP/ADP generation (Müller et al. 2019). ATP has a role in providing energy and molecule transfer channeling maintained by ATP-pumps, contraction of muscle, and building new cellular compartments which require this energy source in many cells. Nutrients are metabolized to liberate energy, which can be stored in phosphoanhydride bonds of ATP. The intracellular ATP concentration (~100 mM) is usually higher than in the ECM (10 nM), and its level varies in the range of 20–100 nM in human blood. Measuring tissue ATP concentration is very difficult.

The ECM does not contain mitochondria, thus its energy level can be fueled without extracellular mitochondria. ATP cannot diffuse through the plasma membrane, but ATP-permeable and ATP-export channels can transport it to the ECM. ATP and polyP are associated with binding proteins in the extracellular space, but no specific ATP/ADP carrier has been identified so far. The roles of extracellular ATP and polyPs were highlighted by the following facts: (a) ATP can be a signaling molecule which is linked to autocrine signaling loop through receptors, such as purinergic receptors (Di Virgilio and Adinolfi 2017); (b) ATP, as a nucleotide, feeds metabolic energy-requiring mechanism in the ECM (e.g., secreted tyrosine kinase in platelet degranulation, phosphorylation by ECM kinases, packaging collagen maturation—peptidyl-trans-isomerases: cyclophylins, FK506-binding proteins, and parvulins)—(Bordoli et al. 2014; Fanghänel and Fischer 2004); (c) ATP can help heat shock protein clustering (e.g., HSP70 is supposed to bind ATP in extracellular space) (Trcka et al. 2019); (d) sol-gel transition theory suggests that this conversion requires free energy (e.g., claudins in tight junctions—(Zhao et al. 2018)); (e) endothelial cell-released ATP can function as a chemoattractant for migrating ring forming cells (Müller et al. 2018a, b); (f) ECM-stored polyPs can be the source for ATP production both intra- and extracellularly. Additionally, there are more evidences that microenvironmental polyPs affect fibroblasts influencing FGF-induced adhesion (Segawa et al. 2011; Shiba et al. 2003). PolyP synthesis is linked to mitochondria that act as a reservoir for metabolic energy, and its degradation is related to alkaline phosphatase (ALP) which can act as polyP transferase. ALPs are membrane-bounded glycoproteins. ALP and adenylate kinase (ADK) inhibitors can block cell migratory behavior, especially in fibroblasts and endothelial cells in different in vitro experiments. Moreover, polyP addition increases and speeds up the migration of endothelial cells demonstrated in scratch tests (Müller et al. 2018a). In these assays, the increased extracellular ATP level is highly modified the migratory behavior of the studied cells. ALP and ADK can phospho-transfer intra- and extracellularly; additionally, polyPs can be transported by exosomes, as well. ECM influences the stability and the degradation of polyPs to charged fibrous proteins (collagen, proteoglycan, and GAGs electrostatic interactions) and their transport mechanisms, but the role of ECM as an energy reservoir has not been described well yet (Müller et al. 2019).

In some studies, organelle transfer can also be detected in cancer cells. In parallel, mitochondrial DNA and mitochondria transfer were confirmed from stromal cells to mitochondrion-deficient cancer cells; these help OXPHOS-dependent survival and adaptations in cancers (Spees et al. 2006; Tan et al. 2015). Based on these transfers, cancer metabolism can be completely rewired depending on the necessities of tumor cell growth.

The increased release of exosomes was also documented in different cancer cells (King et al. 2012; Svensson et al. 2011). It was also suggested that *exosomes* can be enriched in PG-bonded lipoproteins, and in addition, their ligands (e.g., apolipoproteins) could influence cellular communications and *vesicle cargo* (Boussadia et al. 2018; Shao et al. 2018; Parolini et al. 2009). The metabolite composition of exosomes could also modulate cancer cell functions. Hypoxic adipocyte-derived

exosomes and other hypoxic vesicles showed increased accumulations of triglycerides in the tumor microenvironment. These showed higher level of lipogenic enzymes which can be transferred to the neighboring or other distant cells (Sano et al. 2014). It was described that acidic exosome fractions can positively influence the invasive potential of melanoma cells (Peppicelli et al. 2014; Kucharzewska et al. 2013). Additionally, hypoxic vesicles can transfer and mimic hypoxic response in exosome-treated non-hypoxic tumor cells influencing other cellular compartments (Zhao et al. 2016). Based on these, proteins, nucleic acids, and metabolites transported by exosomes through the ECM have to be considered in tumor metabolism and development, as well.

13.5 Complex Metabolic Regulation in the Extracellular Matrix

The continuous changes in ECM influence and regulate the molecule composition of the metabolic niche at different levels: (a) metabolic alterations in tumor cells; (b) metabolic crosstalk between tumor and non-tumor cells (completion and collaboration); (c) metabolic heterogeneity in tumor tissue (e.g., anatomical location of the tumor, tissue-specific metabolites, local hypoxia, and metabolite gradients); (d) homeostasis of the organ, the whole body, and the personal metabolic state (e.g., obesity, diet, systematic glucose-insulin homeostasis, diverse metabolic niches in the body, and gut microbiome-released metabolites) (Elia and Haigis 2021).

The heterocellular collaborations, fibrotic elements, ECM proteins, metabolites, and their metabolic consequences in tissue microenvironment promote tumor growth. Accordingly, tumor cells force many metabolic pathways among normal metabolic processes along with their demand. This summary underlines the consequences of metabolic adaptations, alterations in the constitution of ECM elements. These alterations provide site for metabolic symbiosis, where many players and molecules communicate, compete, cross-feed each other in a way guided by tumor cells to promote tumor cell survival, growth, and if it is necessary to overgrowth non-tumor cell populations, migrate, and metastasize (Figs. 13.1 and 13.2).

These changes together with the exhausting of reserved nutrients and energy forces lead to metabolic collapse in the whole organ and body (additionally, could result in cachexia in the patients). In this multi-stepped evolution, the tumor cells have to proliferate in an organ-specific manner in a special microenvironment. As a consequence, cells undergo adaptation mechanisms and further alterations (e.g., epithelial-mesenchymal transition and degradation of adhesion molecules) to gain migratory and metastatic properties. Metastatic cells have to survive in the blood circulation, under oxidative stress situations, and nutrient depletion. Finally, these cells need to adapt to the new place or reprogram the new microenvironment for maintaining their own growth and survival demand. In these complex processes, especially in tissue environment, cells use matrix elements as frameworks, solid

base, nutrient source, growth cytokine depo, and intercellular communication niche. In this symbiosis, the ECM elements have both passive and active roles including autophagy and exocytosis-mediated feeding; small metabolite exchanges between hypoxic and normoxic cells; LDs and lipoprotein uptake and release of growth factor and cytokine production. In this tumor orchestrated milieu, tumor cells have several advantages (Boedtkjer and Pedersen 2020). Targeting metabolic adaptation mechanisms, metabolic communications and this metabolic symbiosis are in the focus of many recent studies to increase metabolic stress in cancer cells and induce metabolic catastrophe in tumor mass (Anderson et al. 2021; Petővári et al. 2020; Holloway and Marignani 2021). Targeting metabolic vulnerabilities of cancer cells reduce metabolite-derived immunosuppression by enhancing both metabolic fitness and anti-tumor immune functions (Bonglack et al. 2021; Shriwas et al. 2021; Georganaki et al. 2018). The problem is that tissue composition and the ECM are depending on cellular origin, where the local microenvironment influences the metabolic adaptation mechanisms and rewires cancer cell bioenergetics. Moreover, several metabolites and metabolic effects could have certain genetic and epigenetic effects which can modify tumor plasticity. To target metabolic communications in tissue microenvironment, we need to consider many different factors including tumor type, location, stage, and potential metabolic compensatory mechanisms represented in tumor heterogeneity.

However, several recently used drugs with various primary targets affect the ECM as a by-product of the drug actions and metabolites. Until recently, ECM has been less considered in drug discovery. Moreover, the ECM, its composition and elements could also influence drug transport, drug accumulations/concentrations, drug delivery to tumor cells, and finally, the therapeutic effects. Certain recent therapeutics specifically modify the composition of ECM elements: immunomodulatory drugs, chemotherapeutics—e.g., methotrexate and nucleotide synthesis targeting drugs; statins, which alter collagen and proteoglycan expression; non-steroidal anti-inflammatory drugs and mTOR/calcineurin inhibitors modify fibronectin and chondroitin sulfate PGs; antimicrobial antibiotics reduce MMPs production; TK inhibitors reduce the expression of TGFβ-enhanced matrix elements (Järveläinen et al. 2009). Drugs and their metabolites can bind to ECM proteins themselves, and it is also well known that the structure of ECM can often be disrupted as a consequence of therapeutic treatments. It is an alternative strategy to influence the delivery and/or deposition of anti-tumor drugs and their metabolites in the tumoral extracellular matrix. Furthermore, the by-stander effects of these drugs have to be considered to eliminate both tumorous and non-tumorous cells. Adipose compartments, lipids can solve and continuously release lipophyl drugs (e.g., antipsychotic drugs—fluphenazine, olanzapine). Moreover, lipid compartments and their ratio can influence the delivery/blood-brain barrier crossing (e.g., Temozolomide) and the effect of such treatments, as well. There are some drugs that direct target cellular and structural (e.g., Natalizumab, Vedolizumab) elements of the ECM in certain immunological diseases (Schön 2008; Scribano 2018). In conclusion, pharmacological targeting of ECM is a promising way, but more studies are required to evaluate its potential translation to the clinical trials, especially in cancer.

13.6 Concluding Remarks

Cancer metabolism studies have made great progress in describing alterations of cancer and other cells as well as the importance of metabolic niche in tissue microenvironment. Understanding the effects of local metabolite availability and metabolic dependencies in tumor and stromal cells could provide new options in individual therapy using known drugs with metabolic targets. Considering the complexity of tissue heterogeneity and its players including ECM elements, the experimental cancer models and their limits have to be revised in future studies (Lau and Heiden 2020). Hopefully, we will characterize better the metabolic communications, metabolic phenotype of cancer cells, and their important molecular mechanisms in the ECM. In addition, we could target these tumor-promoting alterations more precisely and turn this knowledge to find more benefits for the development of recent personalized therapies.

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Chapter 14
The Role of Inflammatory Cells in Tumor Angiogenesis

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Abstract Tumor growth depends on angiogenesis. The complex tissue environment surrounding tumor cells, which is composed of a variety of resident and infiltrating host cells, secreted factors and extracellular matrix proteins, influences tumor angiogenesis and progression. Moreover, the tumor microenvironment contributes to determining therapeutic responses and resistance to therapy. The ability to block tumor resistance is related to the understanding of the cellular and molecular pathways activated in the tumor microenvironment. Novel emerging targeted therapeutic strategies are based on the combination of different antitumor approaches with the aim of resolving refractory tumors and improving cancer treatment efficiency.

14.1 Tumor Angiogenesis

Healthy and pathologic tissue homeostasis requires an adequate supply of oxygen and nutrients that is connected to efficient development of the vascular system. Additionally, tumor cells to survive and proliferate need oxygen and nutrients and consequently the closeness to blood vessels. Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels (Carmeliet and Jain 2011). Generally, tumor development is an angiogenesis-dependent process, and the angiogenetic process depends on the temporal coordination of factors and related pathways needed for the establishment of stable channels to provide a supply to tumor cells (Weis and Cheresh 2011). It has been well established that during cancer progression, the interactions between tumor cells and inflammatory cells are closely associated with each other and with angiogenesis (Wang et al. 2019a).

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The growth of solid tumor mass, its progression and the metastatic process, how it is widely described, are strongly influenced by angiogenesis (Folkman 1971). In 1966, Warren and collaborators implanted melanoma nodules in experimental animals and observed a rapid vessel sprout toward the mass, the formation of new capillaries, their penetration into the tumor, and the establishment of blood flow. This phenomenon was more evident during tumor growth than in inflammation processes (Warren and Shubik 1966). Research conducted by Folkman showed that without appropriate vascularization and therefore oxygen and nutrient supply, a tumor can grow limitedly to a size of a few millimeters and a cell content of approximately a few thousand cells (Folkman 1971; Nishida et al. 2006). Under these conditions, tumors induce a process recognized as an angiogenic switch in which tumor cells acquire angiogenic properties, leading to the transition from a quiescent to active endothelium and consequently the vascularization of the growing cell mass (Baeriswyl and Christofori 2009; Ribatti et al. 2007). In tumor murine models, this switch coincides with malignant transition of the growing mass and is needed for malignant tumor progression (Lin et al. 2006; Folkman et al. 1989). It became evident that some soluble factors released by the tumor induced the activation of angiogenesis. Folkman hypothesized that until the appropriate blood flow is created, the tumor mass stops its growth and enters a dormant state (Folkman et al. 1971). On this basis, in the last 50 years, research on mechanisms related to tumor angiogenesis has intensified to discover molecules usable as new targets in anticancer therapy. Tumor angiogenesis is a multiphasic process initiated directly by the tumor when it reaches a size that makes it hypoxic, which further leads to cancer development.

14.2 Tumor Microenvironment

It is well known that tumor cells develop in a complex tissue environment, the so-called tumor microenvironment (TME), which includes cancer cells, stromal cells, blood vessels, nerve fibers, extracellular matrix, and acellular components. The TME is involved in tumor initiation as well as during tumor progression and metastasis; furthermore, it also has important effects on therapeutic efficacy (Tamma et al. 2019a). It is believed that although cancer initiation is due to the acquisition of oncogenic mutations in cells, its progression depends on the surrounding cells that are recruited and subsequently release many cytokines and chemokines (Tysnes and Bjerkvig 2007). In 1863, Rudolf Virchow postulated the crosstalk between inflammation and cancer (Virchow 1989), and 20 years after Stephen Paget illustrated the "seed and soil" theory assuming that the choice of the target organ depends on the interactions between metastatic tumor cells (the "seed") and their organ microenvironment (the "soil") (Paget 1989). One hundred years later, Hanahan and Weinberg expanded from six to ten hallmarks of cancer and recognized the important role of the TME in cancer development (Hanahan and Weinberg 2011). The main cytokines and chemokines secreted by cells of the TME are involved in the regulation of

angiogenesis, including proangiogenic factors, such as the vascular endothelial growth factor (VEGF) family, fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), angiopoietins (Ang), and hypoxia-inducible factor (HIF), and angiostatic factors, such as angiostatin, endostatin, platelet factor 4 (PF4), and thrombospondin-1 (TSP1) (Ucuzian et al. 2010).

14.3 Pro-Angiogenic Factors

VEGF The human VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) originating from different genes (Melincovici et al. 2018). The VEGF family exerts its function by binding three transmembrane tyrosine kinase receptors (RTKs), VEGFR-1 (FLT1), VEGFR-2 (KDR, FLK1), and VEGFR-3 (FLT4). VEGFR-1 is expressed in monocytes, macrophages, hematopoietic stem cells, vascular smooth cells, and leukemic cells. VEGFR-2 is expressed in vascular endothelial cells, endothelial progenitor cells, and megakaryocytes, whereas VEGFR-3 is expressed in lymphatic endothelial cells. VEGFs can also interact with other proteins, integrins, cadherins, heparan sulfate proteoglycans, and with the coreceptors neuropilin-1 and -2 (NRP-1 and NRP-2), which enhance VEGFR-1 and VEGFR-2 action (Stuttfeld and Ballmer-Hofer 2009). VEGF-A is the main component of the VEGF family and is produced by endothelial and vascular smooth muscle cells, activated platelets, fibroblasts, lymphocytes, macrophages, and tumor cells. It is considered a crucial angiogenic stimulator involved in numerous pleiotropic effects, including the proliferation and inhibition of apoptosis of vascular endothelial cells (Ferrara and Davis-Smyth 1997; Gerber et al. 1998), permeability, chemotaxis and activation of monocytes and hematopoietic stem cells, and exerts neurotrophic and neuroprotective action (Storkebaum and Carmeliet 2004). Through alternative splicing, the VEGF-A transcript produces several isoforms with proangiogenic or antiangiogenic activities, including VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} (Yang et al. 2018a; Logue et al. 2016; Dehghani et al. 2018). $VEGFA_{165}$ is the most important both quantitatively and qualitatively. VEGF-B is involved in pulmonary angiogenesis after chronic hypoxia and has been found in cardiac and skeletal muscle. VEGF-C and VEGF-D are important lymphangiogenesis regulators (Rauniyar et al. 2018; Stacker and Achen 2018). PlGF, discovered in the human placenta, is highly expressed in trophoblast cells (Hang et al. 2013) and has also been found in the thyroid, lungs, heart, and skeletal muscle (Maglione et al. 1991). It includes four different subtypes that bind VEGFR-1, and the PlGF isoform also binds NRP-1 and NRP-2. PlGF regulates the growth, migration, and survival of endothelial cells directly through VEGFR-1 or indirectly through VEGFR-2/VEGF-A-mediated activation or formation of a PlGF/VEGF-A heterodimer (Autiero et al. 2003).

Fibroblast Growth Factors (FGFs) The human FGF family includes 22 members involved in the regulation of endothelial cell differentiation, proliferation, migration,

survival, and vessel maturation (Yun et al. 2010). FGF-1 and FGF-2, the first known as acid FGF and the latter as basic FGF, mostly mediate the angiogenic response (Motomura et al. 2008). FGF receptors (FGFRs) belong to the RTK superfamily. Upon activation, they undergo dimerization and internalization and initiate large-scale tyrosine phosphorylation responses and signaling cascades activating the Ras/MAP-kinase pathway (Mathew et al. 2016).

Platelet-Derived Growth Factor (PDGF) The PDGF family comprises four PDGF homodimers, namely, PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD, and one heterodimer, PDGF-AB (Fredriksson et al. 2004). PDGF was originally isolated from platelets, but it has been expressed by numerous other cell types, including epithelial and endothelial cells. PDGF receptors (PDGFRs) belong to the family of RTKs and include PDGFRα and PDGFRβ, which are encoded by two different genes (Gao et al. 2018). These receptors are expressed by fibroblasts, pericytes, vascular smooth muscle cells, monocytes, macrophages, lymphocytes, and mast cells and stimulate their proliferation and motility. PDGFs participate in vascular development by acting on the proliferation and survival of vascular mural cells (Olson and Soriano 2011).

Angiopoietins (Angs) The Ang protein family includes four members: Ang-1, Ang-2, Ang-3, and Ang-4 (Lee et al. 2004); the first two are the major members involved in vasculogenesis and vascular repair (Akwii et al. 2019). Angs bind to two receptors belonging to the family of RTKs named Tie1 and Tie2. Tie2 is expressed by endothelial and myeloid cells (Patan 1998). Tie1 is an orphan poorly characterized receptor that seems to be involved in the modulation of Ang/Tie-2 through the formation of heterodimers with Tie-2 (Eklund et al. 2017). Ang-1 is expressed by both mural cells and other nonvascular stromal and tumor cells. It is involved in the regulation of vessel stabilization during embryonic development, vessel remodeling, and maintenance of the normal vasculature (Brindle et al. 2006). Ang-2 is produced by the VEGF-stimulated endothelium, hypoxia, and shear stress, promoting blood vessel wall destabilization through competitive inhibition of Tie-2 and integrin activation. Furthermore, Ang-2 stimulates pericyte detachment, permeability, vascular regression, and lymphangiogenesis (Akwii et al. 2019).

Hypoxia-Inducible Factors (HIFs) HIFs are DNA-binding transcription factors that associate with specific nuclear cofactors under hypoxia (Palazon et al. 2014). They are heterodimers that include both the constitutively expressed HIF-1β subunit and oxygen-regulated HIF-1α or HIF-2α subunit (Hu et al. 2003). In humans, HIF-1α is ubiquitously expressed, while HIF-2α, although it is expressed mainly in the endothelium, in hypoxic conditions, is also expressed in the kidney, pancreas, brain, liver, intestine, and myocardium. When cells are in a hypoxic environment, the hydroxylation process is inhibited, and HIF-α escapes proteasomal degradation, dimerizes with HIF-1β, and associates with transcriptional coactivators (Berra et al. 2001). The latter recognizes hypoxia-responsive genes, resulting in physiological adaptation to hypoxia. Other stimuli, such as nitric oxide and reactive oxygen species (ROS), can also activate HIFs (Wellman et al. 2004).

Many human cancers are characterized by increased levels of HIF, and its expression correlates with mortality (Zhong et al. 1999; Talks et al. 2000). Hypoxic conditions contribute to increased HIF activity, which translates into the regulation of genes involved in angiogenesis, cell survival, metabolism, invasion, and metastasis. In solid tumors, the rapid proliferation of cancer cells limits oxygen diffusion within the tumor, decreasing its concentrations under physiological conditions. This leads to increased expression and activity of HIF, contributing to tumor angiogenesis (Huang et al. 2017; Shi and Fang 2004).

14.4 Angiogenic Inhibitors

Angiostatin Angiostatin is a 38 kDa internal fragment of plasminogen (Cao and Xue 2004; Ji et al. 1998). Angiostatin inhibits endothelial cell proliferation, migration, and tube formation (Pozzi et al. 2000) and induces apoptosis of endothelial cells (Ramirez-Moreno et al. 2020). Moreover, angiostatin inhibits the signaling induced by FGF-2 and VEGF in human microvascular endothelial cells (Redlitz et al. 1999) and inhibits primary tumor growth as well as angiogenesis-dependent growth of metastases (Dell'Eva et al. 2002).

Endostatin Endostatin is an angiostatic 20 kDa internal type XVIII collagen fragment released by proteolytic activity (Wenzel et al. 2006). The hinge region of endostatin contains several proteolytic cleavage sites where matrix metalloproteinases (MMPs), cathepsins, and elastases induce its release and consequently the interaction with cell membrane receptors, including α5β1, αvβ3, and αvβ5 integrin receptors, on endothelial cells (Zatterstrom et al. 2000). Endostatin inhibits the mitogen-activated protein kinase pathway in endothelial cells, leading to the inhibition of angiogenesis (Wickstrom et al. 2005). Endostatin affects VEGF to VEGFR-2 binding and tyrosine phosphorylation (Jia et al. 2004) and inhibits the activities of matrix metalloproteinases-2, -9, and -13 (MMP-2, MMP-9, and MMP-13) (Kim et al. 2000).

Platelet Factor 4 (PF4) PF4 is the most abundant chemokine member of the C-X-C family found in platelets and megakaryocytes. It exhibits antiangiogenic effects both in vivo and in vitro and directly interacts with VEGF-A_{165} (Hang et al. 2013; Maurer et al. 2006).

Thrombospondin-1 (TSP-1) TSP-1 belongs to a family of extracellular matrix (ECM) glycoproteins. TSP-1, initially discovered in platelet granules, is also produced by endothelial cells, monocytes/macrophages, and smooth muscle cells. TSP-1 interacts with numerous ECM proteins, modulates extracellular protease levels, and activates transforming growth factor beta (TGF-β) (Lawler 2002). TSP-1 inhibits angiogenesis by inhibiting the growth, sprouting, and motility of endothelial cells. High concentrations of TSP-1 have the opposite effect, promoting angiogenesis (Lawler and Lawler 2012).

14.5 TME Infiltrating Cells

Macrophages Tumor-associated macrophages (TAMs) are one of the major tumor-infiltrating innate immune cells and play an important role in the TME because they are involved in promoting tumor growth, invasion, metastasis, and therapeutic resistance (Chanmee et al. 2014). TAMs are described in two different polarization states: M1 CD68-positive and M2, CD-163 and CD-206-positive (Medbury et al. 2013). It is generally believed that M1 macrophages are involved in proinflammatory processes by migrating to inflamed tissues and targeting pathogens directly or activating cells of the adaptive immune system. It has been demonstrated that the M1 subpopulation has antitumor function because of its ability to kill tumor cells and recruit cytotoxic T lymphocytes to activate adaptive immune responses (Chanmee et al. 2014). The M2 subpopulation, on the other hand, has the functions of debris removal, angiogenesis stimulation, and tissue reconstruction and promotes tumorigenesis. They induce immune tolerance and attract T regulatory cells and Th2 T cells. It is believed that M2 TAMs have protumor activity because they stimulate angiogenesis and tumor growth (Jayasingam et al. 2019). Usually, TAM recruitment is correlated with the induction of angiogenic switching and is associated with a poor prognosis in most cancer types. Many cytokines and chemokines are secreted by vascular and perivascular cells, stromal cells, and cancer cells that recruit TAMs in the TME and include C-C motif ligand 2 (CCL2), CCL5, CCL7, Ang-2, colony-stimulating factor-1 (CSF1), VEGF, interleukin-33 (IL-33), semaphorin 3D (Sema 3D), endothelial monocyte-activating polypeptide-II (EMAP-II), endothelin (ET)-1 and 2, stromal cell-derived factor 1α (SDF1α/CXCL12), eotaxin, and oncostatin (Wang et al. 2019a).

TAMs can transdifferentiate into vessel-like structures by vasculogenic mimicry. In gliomas, the areas where vascular mimicry is found are characterized by high TAM infiltration and correlated with M2 density (Rong et al. 2016). The angiogenic factors secreted by TAMs include EGF-A, TGF-β, FGF-2, CCL18, Sema4D, adrenomedullin (ADM), and PlGF (Riabov et al. 2014). TAMs express the MCT1-lactate transporter. Furthermore, TAMs express VEGF-A when exposed to hypoxia or in the presence of lactate produced by tumor cells following aerobic or anaerobic glycolysis (Zhang et al. 2020). This effect is mediated by HIF1α, and lactate seems to lead to M2-like polarization of TAMs (Colegio et al. 2014). TAMs have been found to localize frequently in avascular and hypoxic areas of invasive carcinoma of the breast, where the expression of VEGF-A is upregulated (Lewis and Pollard 2006). Fra-1 and the IL-6/JAK/Stat3 signaling pathway in TAMs are involved in the secretion of proangiogenic factors (Choi et al. 2018). TAMs produce CCL18, which stimulates angiogenesis in synergy with VEGF-A (Lin et al. 2015). ADM is a potent vasodilator belonging to the calcitonin superfamily whose secretion by macrophages is upregulated by inflammatory factors and hypoxia. In melanoma, TAM-derived ADM induces angiogenesis in a paracrine manner via the endothelial nitric oxide synthase (eNOS) signaling pathway (Chen et al. 2011). MMP-9, which is highly expressed by M2 macrophages, triggers the angiogenic switch during

carcinogenesis by the release of VEGF-A from the ECM in colorectal cancer (Deryugina and Quigley 2015; Yahaya et al. 2019).

Mast Cells (MCs) MCs are involved in a large spectrum of biological processes, ranging from inflammation and immune modulation to angiogenesis, tissue repair, remodeling, and cancer (Welker et al. 2000). MC precursors complete their differentiation and maturation in target tissues under the control of local growth factors, including IL-9, IL-10, IL-3, IL-4, IL-33, CXCL12, nerve growth factor (NGF), and TGF-β (Hu et al. 2007). MCs are traditionally classified based on the production of tryptase and chymase, and resident MCs of various organs are characterized by the expression and release of peculiar factors related to their tissue-specific functions (Krystel-Whittemore et al. 2015). MCs can be recruited to the tumor microenvironment by tumor cell-released chemoattractants, including stem cell factor (SCF) or CCL-15 (Yu et al. 2018). In the TME, MCs release proangiogenic factors such as FGF2, VEGFA, tumor necrosis factor alpha (TNFα), and CXCL8 (Norrby 2002). Furthermore, they produce MMPs and chymase, and tryptase activates pro-MMPs (Kanbe et al. 1999; Johnson et al. 1998). The localization of MCs in the TME is determined by interactions of CCR2, CXCR2, and CXCR3 with their respective ligands CCL2, CXCL1, and CXCL10. In this way, MCs facilitate tumor angiogenesis and promote tumor invasiveness (Ramirez-Moreno et al. 2020; Komi and Redegeld 2020). On the other hand, numerous cytokines released by MCs contribute to inflammation, inhibiting tumor cell growth and inducing tumor cell apoptosis (Ribatti and Crivellato 2012). MC tryptase activates the Ang-1 pathway and induces endothelial cell proliferation in pancreatic cancer (Guo et al. 2016). MC inactivation delayed the angiogenic switch and malignant progression in early preneoplastic lesion experimental squamous epithelial, intestinal, and pancreatic islet cancer models (Maciel et al. 2015).

Neutrophils Neutrophils release large amounts of soluble factors, including cytokines and chemokines, through which they recruit and activate other immune cells (Malech et al. 2014). Moreover, they are involved in chronic inflammation regulation and in various steps of tumor progression and angiogenesis, exerting both pro-(tumor-associated neutrophil, TAN-N2) and antitumor (TAN-N1) roles. Normal density neutrophils (NDNs) have been associated with cytotoxic antitumor activities, while immature low-density neutrophils (LDNs) exert immunosuppressive protumor activities (Cerecedo et al. 2021). The TME is infiltrated with $CD66b^{+}$ neutrophils, and their number is correlated with poor clinical outcome (Carus et al. 2013). TGFβ reduces endothelial adhesiveness of neutrophils and neutrophil transmigration through the endothelium as well as the number of antitumor neutrophils in the TME (Granot 2019). In a Nod Scid mouse model of human prostate cancer, TANs are the major source of MMP-9 (Li et al. 2020a). In gliomas, the high TME infiltration of neutrophils was correlated with the tumor grade as well as resistance to anti-VEGF therapy (Liang et al. 2014). Neutrophils produce low amounts of tissue inhibitors of metalloproteinases-1 (TIMP-1), thus enhancing the angiogenic effect of MMP-9 (Wang et al. 2019b). In a RIP-Tag murine model, granulocyte-CSF (G-CSF) stimulates neutrophils to release the proangiogenic molecule Bv8, which is critical

for VEGF-independent tumor angiogenesis (Bjornmalm et al. 2017). Resistance to anti-VEGF therapy in tumors has been correlated with the infiltration of neutrophils and associated with Bv8 neutrophil expression (Shojaei et al. 2008). On the other hand, neutrophils are also involved in the inhibition of angiogenesis through the release of antiangiogenic factors, such as affecting neutrophil migration toward CXCL1 and CXCL8 (Jeronimo et al. 2017).

Lymphocytes The role of lymphocytes in tumor progression and angiogenesis remains to be further explored, and conflicting data about their function in the TME are emerging (Paijens et al. 2021). B cells are often present in the TME, and it is believed that they may contribute to tumor angiogenesis via STAT3 activation. STAT3 activation in cancer promotes tumor cell survival and proliferation, and a positive correlation has been established between its expression and VEGF release (Yang et al. 2013). It is thought that although only a subset of B cells infiltrating the tumor express STAT3, this might be enough to potentiate and maintain persistent STAT3 activation. Transplantation of STAT3-expressing B cells in tumor mouse models increased tumor growth and angiogenesis through the production of VEGF (Wang et al. 2019c). Another way by which B cells contribute to tumor angiogenesis is the antibody-mediated activation of Fcγ receptors on TAMs. This mechanism induces the secretion of IL-1, leading to the recruitment of myofibroblasts and promotion of tumor angiogenesis (Voronov et al. 2014). Tumor-infiltrating T cells play an important role in the antitumor response by the production of many cytokines, such as TNF-α, interferon gamma (IFN-γ), IL-2, IL-17, IL-22, and IL-36. TAMs inhibit $CD8^+$ T-cell infiltration and antitumor function (de Ruiter et al. 2017; Lan et al. 2021). Regulatory T (Treg) cells are immunosuppressive cells that affect the specialization and function of antigen-presenting cells (APCs), decrease their interactions with T cells, and subsequently inhibit effector T-cell function (Maimela et al. 2019). In addition, Tregs suppress natural killer (NK) cell activities (Li et al. 2020b). Cytotoxic T cells in the TME release IL-2, IL-12, and IFN-γ, improving the cytotoxic functions of $CD8^+$ T cells through the production of TNF-related apoptosis-inducing ligands (TRAILs), ROS, and perforin (Grossman et al. 2004). Tumor cells express coinhibitory receptors such as programmed death ligand-1 (PD-L1) and CD80 that interact with the inhibitory molecules programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed by $CD8^+$ T cells. These interactions may inhibit $CD8^+$ T-cell activation and function (Cai et al. 2019). $CD4^+$ and $CD8^+$ T cells produce FGF-2 and heparin-binding epidermal-like growth factor (HB-EGF), which are both proangiogenic factors (Blotnick et al. 1994). On the other hand, T cells are also involved in the antiangiogenic response through TNFα, TGFβ, and IFNs. IFNs induce the expression of CXCL-9, CXCL-10, and CXCL-11 with angiostatic activities that can directly bind CXCR3 on endothelial cells (Blotnick et al. 1994; Beatty and Paterson 2001). NK cells are able to control tumor growth through their cytotoxic activity (Wu and Lanier 2003). Intratumor NK cells display phenotypic and/or functional alterations compared with peripheral NK cells depending on the influence of local factors and/or the interaction with other cell types of the TME (Larsen et al. 2014). The presence of TGF-β inhibits CD16,

perforins, granzymes, and IFN-γ secretion, reverting NK cells to a proangiogenic phenotype characterized by the secretion of VEGF. Furthermore, the interaction between the immunoregulatory class I MHC molecule HLA-G and the KIR2DL4, ILT-4, and ILT-2 inhibitory NK cell receptors induces NK cells to acquire proangiogenic activities. Prostaglandin E2 (PGE2) is believed to contribute to the NK cell angiogenic switch (Bassani et al. 2019). Tumor-infiltrating NK cells express high levels of CD56, but low levels or none of CD16 produce several factors, such as VEGF, angiogenin, Ang-1, PIGF, CXCL8, and MMPs, which stimulate endothelial cell growth and angiogenesis (Bruno et al. 2018).

Cancer-Associated Fibroblasts (CAFs) CAFs are able to interact with tumor cells and form a myofibroblastic microenvironment that supports tumor progression and angiogenesis via secretion of various growth factors, cytokines, chemokines, and the degradation of ECM (Liu et al. 2019). A significant percentage of CAFs may share endothelial markers such as PECAM/CD31, and this allows us to suppose that they originate from an endothelial subpopulation through endothelial-to-mesenchymal transition (Potenta et al. 2008). Regarding their influence on angiogenesis, several studies have shown that their secretome is rich in several cytokines with proangiogenic effects, including VEGF, CXCL-8, and FGFs (Linares et al. 2020). Furthermore, CAF release is able to form capillary-like structures through vasculogenic mimicry by TGF-β and SDF-1 paracrine action (Yang et al. 2016a). Moreover, SDF-1 recruits endothelial precursor cells (EPCs), which may transdifferentiate into endothelial cells and stimulate the formation of novel vasculature at the tumor-host cell interface (Orimo et al. 2005). CAFs express podoplanin, which promotes angiogenesis in breast cancer via upregulation of VEGF-C rather than VEGF-A or VEGF-D (Kubouchi et al. 2018). The galectin family of glycan-binding proteins displays important functions in cancer development and progression. In gastric cancer, CAF expression of Galectin-1 is upregulated, leading to enhanced VEGF expression. Under hypoxic conditions, G-protein-coupled estrogen receptor (GPER) downregulation in CAFs reduces VEGF expression (Ham et al. 2019). In human pancreatic adenocarcinoma, VEGF expression by CAFs may be regulated by fibroblast activation protein α (FAP α), which is involved in affecting the balance of pro- and anti-angiogenic mediators (Higashino et al. 2019).

14.6 TME Inflammatory Cells and Angiogenesis. Our Experience in the Study of Human Lymphomas

We studied the inflammatory cell infiltrate and its role in tumor angiogenesis in diffuse large B-cell lymphoma (DLBCL) by comparing activated B-cell-like (ABC) patients to germinal center B-cell-like (GCB) patients. We demonstrated that increased ABC expression of STAT3 was correlated with poor prognosis in DLBCL and was associated with higher M2 TAM (Fig. 14.1a, b) and $CD8^+$ (Fig. 14.1c, d) cell infiltration into the TME, which, in turn, induced a strong

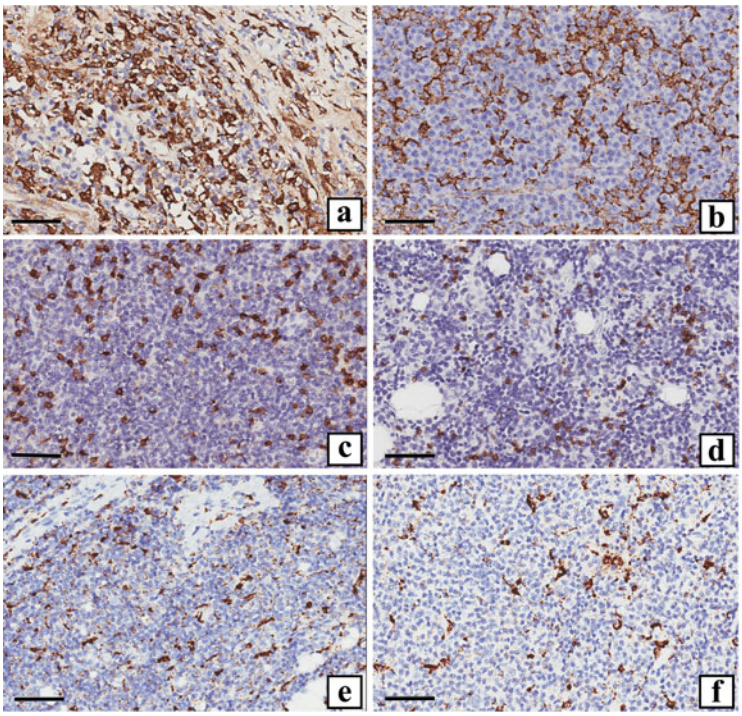

Fig. 14.1 Immunohistochemical staining of $CD163^+$ macrophages in ABC (**a**) and GCB (**b**) DLBCL samples; $CD8^+$ T cells in ABC (**c**) and GCB (**d**) DLBCL samples; $CD68^+$ macrophages in MALT lymphoma (**e**) and control (**f**) samples. Scale bar 60 mm

angiogenic response in the ABC group (Tamma et al. 2020). Moreover, tumor vessels appeared lined by endothelial cells expressing both FVIII and STAT3 (Tamma et al. 2019b). Regarding the morphological distribution of the different TME cells in DLBCL, we established that cell patterns generated by $CD4^+$, $CD8^+$, $CD68^+$, $CD163^+$, and $tryptase^+$ mast cell profiles have a higher uniformity index in the ABC, indicating a tendency of the cells to assume a more uniform distribution in the tissues in this more aggressive DLBCL subtype (Guidolin et al. 2021). Recently, Laddaga and coworkers suggested that the number of tumor infiltrating lymphocytes in the DLBCL TME is connected to a pre-existing antitumor immune response and then to an improved therapy response (Laddaga et al. 2021).

In a further study, we demonstrated that mucosa-associated lymphoid tissue (MALT)-type lymphoma and the tumor inflammatory TME included a high number of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes, $CD68^+$ (Fig. 14.1e, f), $CD163^+$ macrophages, and $tryptase^+$ mast cells. Interestingly, $CD8^+$ cell content positively correlated with both $CD34^+$ vessels, remarking on the important role of these cells in tumor angiogenesis and with $CD163^+$ TAMs. Moreover, $tryptase^+$ mast cells correlated with $CD4^+$ lymphocytes (Tamma et al. 2021).

14.7 Targeting Angiogenesis and Inflammatory Cells in TME

Chemotherapy associated with surgery and/or radiotherapy is the principal cancer therapy worldwide (Bjornmalm et al. 2017). The TME has been gradually recognized as a crucial contributor to cancer progression and drug resistance (Heinrich et al. 2012), so the study of the components of the TME was deepened to identify new therapeutic targets.

Targeting Angiogenesis Bevacizumab was the first anti-VEGF antibody Food and Drug Administration (FDA) approved and actually used in different cancers, including metastatic colorectal cancer, lung cancer, kidney cancer, glioblastoma metastasis, and HER2-negative breast cancer, with response rates and durations highly variable (Jang et al. 2017). The addition of bevacizumab to chemotherapy has shown improvements in progression-free and overall survival with respect to chemotherapy alone (Jang et al. 2017; Yang et al. 2017). Another strategy consists of the inhibition of VEGF binding to its receptors by soluble decoy receptors (Holash et al. 2002). Aflibercept is a recombinant fusion protein containing portions of human VEGFR-1 and VEGFR-2 extracellular domains fused to the Fc portion of human immunoglobulin G1 able to bind with high-affinity VEGF and PlGF, inhibiting the activation of cognate VEGFRs (Holash et al. 2002). Experimental data about the use of aflibercept in cancer xenograft models demonstrated greater antitumor activity than bevacizumab (Chiron et al. 2014). Ramucirumab is a monoclonal anti-VEGFR-2 antibody used as monotherapy or in combination with paclitaxel for the treatment of advanced gastric or gastroesophageal junction adenocarcinoma, metastatic non-small cell lung cancer (NSCLC), and colorectal cancer (Singh and Parmar 2015; Aprile et al. 2014). Tyrosine kinase inhibitors (TKIs) are used for the inhibition of VEGFRs, PDGF-A and PDGF-BRs, and c-Kit (Hamberg et al. 2010; Wang et al. 2016). Among TKIs, pazopanib is commonly used for the treatment of advanced renal cell carcinoma and soft tissue sarcoma (Hamberg et al. 2010; Nakano et al. 2019) and sunitinib is used in metastatic renal cell carcinoma (Roma-Rodrigues et al. 2019). Sunitinib has more benefits than sorafenib as a first-line therapy, although sunitinib has higher toxicity than sorafenib (Deng et al. 2019). M-TOR inhibitors decrease endothelial cell proliferation through the mTOR/AP-1/VEGF pathway, among which everolimus (Wang et al. 2016). Patients treated with antiangiogenic agents have a reduced response to therapies for the acquisition of drug resistance. Two mechanisms of this resistance are the activation of alternative signaling pathways and the upregulation of alternative angiogenic factors and cytokines. Deepening these pathways would allow us to elaborate new treatments and the development of combination regimens with more durable clinical benefits (Philips and Atkins 2014). Anti-VEGF treatment in pancreatic cancer induces increased expression of FGF-1 and -2 and Ang-1 (Zhuang et al. 2010). In patients affected by colorectal cancer treated with bevacizumab, high levels of Ang-2 were detectable (Goede et al. 2010). In glioblastoma multiforme, anti-VEGFR therapy

leads to increased levels of FGF-2 and SDF-1. Similar results have been found in lung cancer models resistant to angiogenesis inhibitors in which epidermal growth factor receptors (EGFRs) and FGFRs are overexpressed (Cascone et al. 2011). In colorectal and renal cancer patients treated with TKIs, increased levels of PIGF and VEGF were detectable (Motzer and Bukowski 2006). Vanucizumab, a bispecific anti-Ang-2/anti-VEGF-A antibody, revealed an acceptable safety profile and promising antitumor activity (Hidalgo et al. 2018). FGFR inhibitors restore the sensitivity to bevacizumab in tumor mouse models (Gyanchandani et al. 2013), but further research failed to determine the relevance of this association (Norden et al. 2015; Semrad et al. 2017). The VEGFR, FGFR, and PDGFR multiple receptor TKI lenvatinib showed promising effects in several tumors and should be considered for counteracting resistance to antiangiogenic agents (Suyama and Iwase 2018).

Anti-angiogenic therapies induce the production of cytokines, such as SDF1, IL-8, and G-CSF, involved in the recruitment of bone marrow-derived cells (BMDCs), which contributes negatively to the anti-angiogenic effect (Montemagno and Pages 2020). An increase in $CD11b^{+}$ $Gr1^{+}$ myeloid-derived suppressor cells (MDSCs) has been observed in tumors not sensitive to anti-VEGF-A treatment (Shojaei et al. 2007). Th-17 cells induce the expression of G-CSF by CAFs and consequently the recruitment of MDSCs (Shojaei et al. 2009). Hypoxia has been related to sunitinib resistance in glioblastoma and breast and metastatic renal cell carcinoma as a consequence of the increased recruitment of MDSCs to the tumor niche (Piao et al. 2012).

Vessel co-option is believed to be correlated with refractoriness to anti-VEGF drug treatment of colorectal cancer liver metastases (Frentzas et al. 2016) and has been observed following anti-VEGFR-2 inhibition in cerebral melanoma metastases (Frentzas et al. 2016). Moreover, vessel co-option has been evidenced in human breast cancer liver metastases, NSCLC, and lung metastases (Kuczynski et al. 2016). The blockade of both VEGF-A and ARP2/3, VEGFA and c-MET or VEGF-A and ZEB2 suppresses vessel co-option and tumor invasion (Sennino et al. 2012; Depner et al. 2016). Vasculogenic mimicry is deeply associated with poor patient survival (Sun et al. 2004). In ovarian cancer models, bevacizumab may induce the progression of metastatic disease, which would correlate with a hypoxic response and vasculogenic mimicry (Xu et al. 2012). Studies on the TME in everolimus-resistant renal carcinoma demonstrated that the antiangiogenic drug stimulates vasculogenic mimicry by differentiating tumor cells into endothelial-like cells (Serova et al. 2016). Moreover, everolimus induces triple-negative breast cancer invasion via vasculogenic mimicry; thus, its evaluation could be helpful in predicting the efficacy of antiangiogenic therapy in these patients (Sun et al. 2017).

Targeting TAMs Targeting TAM-recruiting mediators, which include chemokines, complement components, CSF-1, and VEGF, is being studied (Liu et al. 2020). It has been reported that the inhibition of CSF1R in glioblastoma and cervical and breast cancer murine models induces a dramatic reduction in tumor volume and survival of mice (Pyonteck et al. 2013). This inhibition seems to reprogram TAMs by GM-CSF to induce their repolarization to an antitumoral

state (Quail and Joyce 2013; DeNardo et al. 2011). The monoclonal antibody RG7155 in human patients led to a remarkable reduction in CSF-1R$^+$ CD163$^+$ macrophages in diffuse-type giant cell tumor patients (Ackermann et al. 2013). TAM reduction improves antiangiogenic treatments. Treatment with vascular-disrupting agents such as combretastatin-A4-phosphate has been reported to markedly increase its efficacy when TIE2$^+$ TAM recruitment is blocked (Welford et al. 2011). The reduction in TAMs augmented the effects of sorafenib (Zhang et al. 2010). In addition, TAMs improved the antiangiogenic and antitumor effects of VEGF/VEGFR2 antibodies in subcutaneous tumor models (Priceman et al. 2010). TAMs limit the cytotoxic activity of CD8+ cytotoxic T cells during tumor progression, mainly in the M2 polarization state. Inhibiting TAM recruitment or blocking TAM polarization to the M2 phenotype may enhance T-cell-mediated antitumor responses and improve the efficacy of immunotherapies (Coussens et al. 2013). Moreover, some immunotherapies may also depend on the reprogramming of TAMs toward an M1 phenotype. One method used to reprogram TAMs is histidine-rich glycoprotein (HRG) treatment, which induces macrophage downregulation of PlGF and stimulates the normalization of blood vessels and the efficiency of chemotherapy in mouse tumor models (Rolny et al. 2011). Other strategies include the suppression of nuclear factor-kB signaling (Hagemann et al. 2008) or exposure to anti-IL-10R antibodies combined with the TLR9 ligand CpG (Guiducci et al. 2005).

Targeting TANs Inhibition of the protumor functions of TANs (Hsu et al. 2020) may be combined with conventional or new anticancer therapies to improve the antitumor effects (Khan et al. 2020). CXCR2 inhibitors are also used in combination with other therapies in clinical evaluation in patients with different tumors (Li et al. 2019; Timaxian et al. 2021; Groth et al. 2021; Cabrero-de Las Heras and Martinez-Balibrea 2018). The neutrophil-derived enzyme elastase promotes tumor growth and invasiveness. The elastase inhibitor ONO-5046 reduced tumor growth in NSCLC (Houghton et al. 2010). Another approach has been to reprogram neutrophil function in the TME through the inhibition of TGFβ (Qin et al. 2020). The inhibition of angiotensin-converting enzyme and the angiotensin II type 1 receptor nicotinamide phosphoribosyltransferase (NAMPT) or CXCR4 is another approach to reprogram neutrophils to an antitumor state (Shrestha et al. 2016; Yang et al. 2018b).

Targeting CAFs The protein FAP is considered a candidate for targeting CAFs because it is expressed in tumors but not in healthy tissues and is considered a predictor of poor survival (Liao et al. 2013). Nevertheless, both sibrotuzumab, an antibody targeting FAP, and inhibitors of FAP activity induced lower survival rates (Liu et al. 2019; Yang et al. 2016b). An IL-2 variant targeting FAP, RO6874281, is under investigation (Joshi 2020; Koustoulidou et al. 2021).

14.8 Concluding Remarks

Cytokines and chemokines secreted by cells of the TME are involved in the regulation of tumor angiogenesis based on the balance of pro- and antiangiogenic factors. Deepening the mechanisms underlying the crosstalk between the TME and tumor cells has allowed the discovery of numerous molecular-targeted drugs that control diverse elements of the TME. Different approaches varying from traditional and emerging inhibitors of angiogenic cytokines and their receptors to the modulation of TME cell activities and novel immune checkpoint inhibitors proved to be promising in tumor progression. Despite the promising results of these new therapeutic approaches, their efficacy is often limited by evasion, and resistance mechanisms have emerged. Overcoming resistance to antitumor therapies is a great challenge but might lead to the improvement of the clinical outcome of patients and, for this reason, currently constitutes a major focus of research.

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Chapter 15
Cancer Angiogenesis and Its Master Regulator Perlecan

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

Abstract Angiogenesis and vasculogenesis are not only indispensable for normal tissue growth and regeneration but also particularly vital for tumors. As without sufficient blood supply that provides nutrients, tumor cells cannot proliferate. Tumors may employ several strategies to receive sufficient blood supply: they can promote the development of new blood vessels, engulf already existing arteries, or induce the splitting or glomeruli-like proliferation of pre-existing blood vessels. The outcome depends on the equilibrium of pro- and antiangiogenic factors that regulate these events. The most potent stimulatory factor is VEGF, but several other growth factors, cytokines, chemokines, and miRNAs have a stimulatory potential, whereas specific proteolytic breakdown products and other miRNAs can inhibit angiogenesis. Like tumor cells, newly generated tumor vasculature also displays deregulated behavior: blood vessels created upon the angiogenic switch differ from their normal counterparts in both structure and function. *Perlecan*, a large extracellular matrix heparan sulfate/chondroitin sulfate (HS/CS) proteoglycan, is a ubiquitous component of basement membranes, including vascular basement membranes of both normal and tumorous blood vessels. Besides its structural role, perlecan regulates angiogenesis by interacting with a large number of partner molecules, mainly with growth factors and cytokines bound to its HS chains. Whereas intact perlecan promotes angiogenesis, a tryptic fragment of its domain V called endorepellin exerts anti-angiogenic action and triggers tumor autophagy, whose potentials may be exploited in the future management of cancer.

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15.1 Cancer Angiogenesis

Since the first publications on cancer angiogenesis appeared more than 20 years ago, anti-angiogenic therapies, with the aim to deteriorate tumor blood supply, have emerged as leading approaches toward conquering cancer. To obtain sufficient nutrition and oxygen, rapidly proliferating cancer cells require an increased blood supply (Folkman 1971). However, no trivial physiological mechanism is available to fulfill this demand. Therefore, tumor cells need to deploy different strategies depending on their type and localization and utilize multiple factors to trick stromal components into forming new blood vessels. The last decades have seen intensive efforts to clarify the critical steps of tumor angiogenesis, a key prerequisite to cancer cell proliferation and invasion (Pluda 1997). Research has elucidated a number of typical pathways that characterize tumor angiogenesis (Döme et al. 2007). (*1*) Tumors can facilitate angiogenesis by promoting the development of new capillary buds from already available blood vessels. This process, also referred to as sprouting, is supported by a series of stimulatory factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF) beta, matrix metalloproteases (MMPs), and integrins (Bergers and Benjamin 2003; Mehrzadi et al. 2021). (*2*) Tumor tissue can surround and engulf preexisting blood vessels, a process typically observed in liver metastases (Latacz et al. 2020). (*3*) A further approach to increase the number of tumor-supplying blood vessels is the so-called intussusception, whereby preexisting blood vessels split up via ingrowth of a transmural pillar (De Spiegelaere et al. 2012). (*4*) A special form of angiogenesis characteristic of glioblastoma multiform is glomeruloid angiogenesis, whereby preexisting blood vessels (Brat and Van Meir 2001) form convoluted loops in response to the pulling force from proliferating tumor cells (Lugano et al. 2020).

15.2 Critical Participants of Tumor Angiogenesis

The development of blood vessels requires a set of structural and regulatory factors. This is also true for the different forms of tumor angiogenesis discussed above (Fig. 15.1).

15.3 Structural Compartments of Tumor Blood Vessels

(a) *Endothelial cells.* Contrary to the historical view of tumors, abnormal phenotype is not restricted to cancer cells but also characterizes stromal cells including vascular endothelial cells (Hida et al. 2018, 2010, 2016). Upon modulation by the tumor microenvironment, these cells change their phenotype, express new

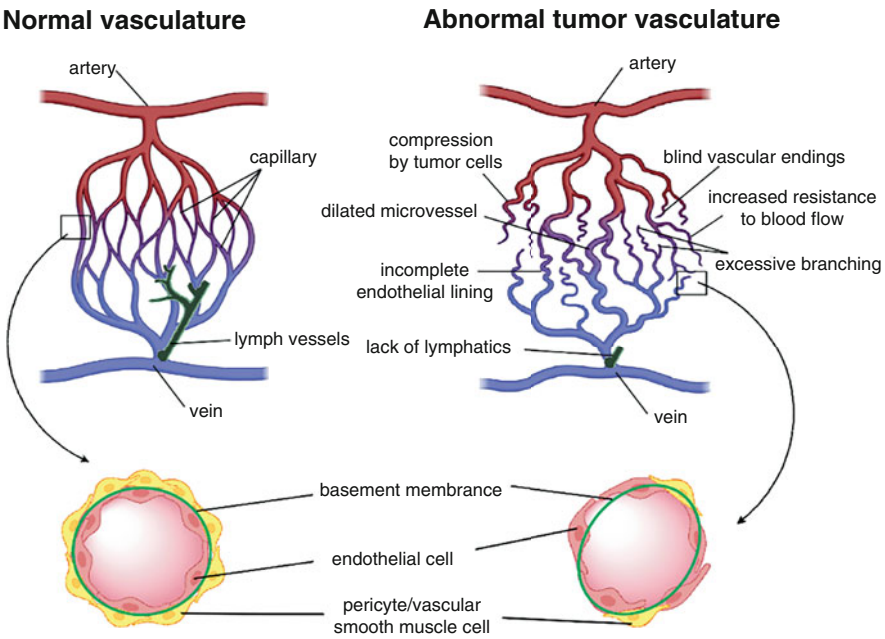


Fig. 15.1 Tumor vasculature is characterized by a disorganized arrangement which involves loss of the well-structured fashion of gradually decreasing diameter of the arteries ending in the branching capillary system followed by the collection of deoxygenized blood in the venous side, always detectable in normal situation. Instead, leaky blood vessels with uneven diameters, insufficient to withstand the vascular pressure, small arteries with dead ends, incomplete endothelial linings, uneven arrangement of pericytes, and incomplete basement membranes can be observed

cell surface markers (St Croix et al. 2000; Høye et al. 2018), and display altered response to regulatory factors (Amin et al. 2006; Matsuda et al. 2010). Changes may encompass increased angiogenic capacity and drug resistance (Xiong et al. 2009).

(b) *Pericytes* in tumor microvessels are arranged abnormally loosely on the abluminal side of endothelial cells, display irregular shape, and possess aberrant cytoplasmic processes. Therefore, they fail to provide adequate support to the tumor neovasculature (Raza et al. 2010; Armulik et al. 2005).

(c) *Basement membrane* is also irregular, with varying thickness and loose association to the endothelial cells. These abnormalities result in leaky blood vessels surrounded by small pools of extravasated blood (Baluk et al. 2003; Iozzo et al. 2009; Zuazo-Gaztelu and Casanovas 2018).

15.4 Regulatory Factors of Tumor Angiogenesis

15.4.1 Tumor-Associated Stromal Cells

Bone marrow-derived stromal cells are implicated in the production of regulatory factors that promote or inhibit blood vessel generation. Neutrophils and eosinophil granulocytes, monocytes, and tumor-associated macrophages (TAMs) migrate to the tumor and secrete angiogenesis modulators like VEGFA, FGF2, and MMPs. TAMs also produce chemokines with pro- or antiangiogenic potential and interleukins with proinflammatory effect (Cassetta and Pollard 2020; Chen et al. 2019; Stockmann et al. 2014; Lin et al. 2006; Owen and Mohamadzadeh 2013). Lymphocytes actively participate in local immune reactions and may inhibit angiogenesis and tumor proliferation by interfering with the effect of PD-L1 (Asadzadeh et al. 2017; Tian et al. 2017; Thienpont and Lambrechts 2017).

15.4.2 Tumor-Associated Fibroblasts (TAFs)

Whereas normal fibrocytes do not synthesize angiogenic factors, TAFs transformed from normal fibrocytes as well as from bone marrow stem cells engage in the production of VEGF upon stimulation from cancer cells (Wang et al. 2019); VEGF, in turn, may promote either vasculogenesis or angiogenesis. This interaction is likely to be bidirectional, as the conditioned medium of TAFs facilitates VEGF production of ovarian cancer cells in culture (Xu et al. 2013), and IL-6, the major stimulator of VEGF secretion, is produced by both the tumor and TAF cells. Fibrocytes can also gain TAF phenotype as an effect of IL-6. In addition to VEGF, TAFs can synthesize FGF2, PDGF, and CXCL12, all of which promote tumor angiogenesis (Wang et al. 1999; Cao et al. 2008) (Table 15.1).

Table 15.1 Representative regulatory factors of angiogenesis

Pro-angiogenic factors	Anti-angiogenic factors
VEGF	Angiostatin
FGF	Endostatin
PDGF	Endorepellin
EGF	Canstatin
HGF	Tumstatin
Angiopoietin	Thrombospondin
TGF-beta	
CXCL12	

15.4.3 Proangiogenic Factors

Under physiological conditions, a delicate balance of pro- and antiangiogenic factors results in quiescence of blood vessels. This equilibrium breaks down in tumors as rapidly proliferating cancer cells require more blood and O_2 supply, thus together with stromal cells start to secrete angiogenesis-stimulating factors. While VEGF is the best known among them, there are many more mediators that trigger the proliferation of tumor blood vessels.

The VEGF family contains four members: A, B, C, and D; among these, VEGFA is the most important. VEGFA binds to its cell surface tyrosine kinase (TK) receptors VEGFR1 and VEGFR2 to stimulate the proliferation of endothelial cells (Holmes and Zachary 2005; Leone et al. 2019). Among the members of the FGF family, basic FGF (bFGF, FGF2) is the most potent stimulator of angiogenesis both in normal and tumor-induced blood vessels. bFGF is secreted by both tumor and stromal cells. In cooperation with VEGF, they collectively promote the secretion of proteases that facilitate the degradation of ECM and support the invasion of cancer cells (Turner and Grose 2010; Lugano et al. 2020). A plethora of data suggests that in addition to VEGF and FGF, PDGF is also implicated in both normal and tumor-associated angiogenesis. In tumors, PDGF signals through the PDGF receptor. Endothelial cells of tumor blood vessels secrete PDGF, while pericytes express PDGF receptor, thus promoting their own recruitment to the tumor blood vessels. PDGF stimulates both angiogenesis and cancer cell proliferation, and decreases intratumoral pressure (Raica and Cimpean 2010).

By signaling through the TK receptor Tie2, angiopoietin 2 also acts as a potent driver of tumor angiogenesis and its increased expression generally indicates poor tumor prognosis. Angiopoietin 2 mobilizes myeloid cells which, once transformed into stromal cells, promote tumor progression and downregulate tumor immunity (Yu and Ye 2020). Furthermore, by signaling via integrinβ1, angiopoietin 2 induces the expression of MMPs, which can destabilize the vasculature (Etoh et al. 2001). MMP2, MMP9, and MMP14 represent all active contributors to the angiogenic switch as matrix degradation increases the bioavailability of angiogenic factors (Bergers et al. 2000). At the same time, the degradation products of matrix components may exert anti-angiogenic effects, like in the case of endostatin and angiostatin (O'Reilly et al. 1999).

Hypoxia induced by insufficient blood supply inhibits the proteasomal degradation of hypoxia-inducible factor1α (HIF1α). HIF1α enters the nucleus and by associating with HIF1β it forms a heterodimeric transcriptional complex that binds to hypoxia-responsive elements. This initiates the synthesis of a plethora of proteins involved in angiogenesis including VEGF, nitric oxide synthase (NOS), leptin, erythropoietin (EPO), and many others (Masoud and Li 2015). Chemokines and their receptors have also been shown to play an active role in angiogenesis and also several members of the CXC and CXCL families possess an angiogenesis-stimulating potential. CXCL2, 3, 5, 6, 7, and 8 stimulate angiogenesis by binding to the endothelial cell surface receptor CXCR2. CXCL8 is one of the most potent

chemokines that facilitates angiogenesis in ovarian, lung, and colon carcinoma, as well as in melanoma (Singh et al. 2007). Also other chemokines can exert an angiostatic effect (Sozzani et al. 2015; Belperio et al. 2000; Strieter et al. 2006).

Tumor cells may shed small cytoplasmic vesicles called exosomes into the bloodstream. Among other cargoes, tumor-derived exosomes may carry miRNAs and long noncoding RNAs. In the past 10 years, it has been increasingly recognized that these miRNAs may affect the action of tumor angiogenesis regulators, e.g., pro-angiogenic potential has been reported for miR155 (VHL), miR566 (VHL), miR210 (EFNA3), miR21(PTEN), miR182, miR296, miR17-92, miR378, miR221/222, and let-7b/-7f (Arcucci et al. 2021).

15.5 Natural Inhibitors of Angiogenesis

15.5.1 Proteolytic Breakdown Products

Endostatin, angiostatin, and endorepellin exemplify a peculiar antiangiogenic mechanism. All three molecules are degradation products of proteins that in their native form fulfill a completely different function. Angiostatin is derived from plasminogen, endostatin is the tryptic fragment of collagen XVIII, while endorepellin corresponds to the domain V of perlecan. Interestingly, angiostatin on its own has only a modest effect on endothelial cells; instead, it targets the innate immune system and utilizes the cytokine IL-12 to mediate its inhibitory effect (Albini et al. 2009). The beneficial effect of IL-12 was confirmed in a model of pathologic angiogenesis as well (Zhou et al. 2016). In contrast with angiostatin, endostatin directly targets endothelial cells by binding to Frizzled and VEGF receptors, as well as to glypicans (Poluzzi et al. 2016). The binding of endostatin triggers diverse downstream events mediated by intracellular pathways involved in angiogenesis such as MAPK, Hif1α, NFκB, STAT, etc. (Abdollahi et al. 2004); consequently, endostatin inhibits angiogenesis and induces autophagy. The activity of endorepellin overlaps with that of endostatin; it also utilizes VEGF receptors, whereas it interacts with different integrins (Kapoor et al. 2020).

Five members of the *thrombospondin* (TSP) family are all large multidomain proteins of the ECM. Their major representative is TSP1 with several functions including anti-angiogenesis. It inhibits the proliferation, migration and survival of endothelial cells, induces their apoptosis and antagonizes VEGF (Lawler and Lawler 2012). This effect is based on the interaction of its so-called TSR (thrombospondin type I repeat) domain with the cell surface proteins CD36, CD47(IAP), and integrins on endothelial cells. The TSR domain also sequesters active MMP2 and MMP9, thereby hindering the release of VEGF from the ECM (Bein and Simons 2000; Rodríguez-Manzaneque et al. 2001). Whereas low levels of TSP were originally thought to be associated with poor tumor survival, and TSP overexpression was linked with delayed angiogenesis, recent publications have reported contradictory data (Huang et al. 2017). Thus, it seems that the effect of TSP1 also depends on other

microenvironmental factors. Besides TSP1, the potential of TSP2 to inhibit tumor angiogenesis has been also reported, which effect could be reversed by miR-221-3p (Wu et al. 2019).

Tumstatin is a 28-kDa cleavage product of type IV collagen, a major constituent of basement membranes. Via signaling through αvβ3-integrin, tumstatin induces apoptosis of vascular endothelial cells, inhibits the synthesis of mTOR pathway proteins, and prevents the dissociation of eIF4E from the 4E binding protein (Maeshima et al. 2002). Similar to tumstatin, *arresten* and *canstatin* are also derived from type IV collagen (Mundel and Kalluri 2007). They also suppress tube formation, proliferation, and invasion of endothelial cells, and promote their apoptosis. Both signal via integrin receptors (Aikio et al. 2012; Panka and Mier 2003) and canstatin induce FAS-dependent apoptosis of endothelial cells.

15.5.2 Noncoding RNAs

As mentioned above, certain noncoding RNAs may promote or inhibit angiogenesis. Some may even behave in a context-dependent fashion, e.g., miR221/222 can act in either direction depending on the tissue (Khella et al. 2015; Nicoli et al. 2012). These miRNAs most commonly target VEGF (Kong et al. 2016; Xue et al. 2016) (mir121, 122, 126, 29b, 206, 140-5p, 497, 377, 134), but effects have been reported on several other molecules implicated in angiogenesis such as VEGFR1,2, HIF1α, MMP2, ETS1, STAT3, STAT5A, CD44, and the Akt-mTOR pathway (Fang et al. 2011; Jeyapalan et al. 2011; Chen et al. 2016).

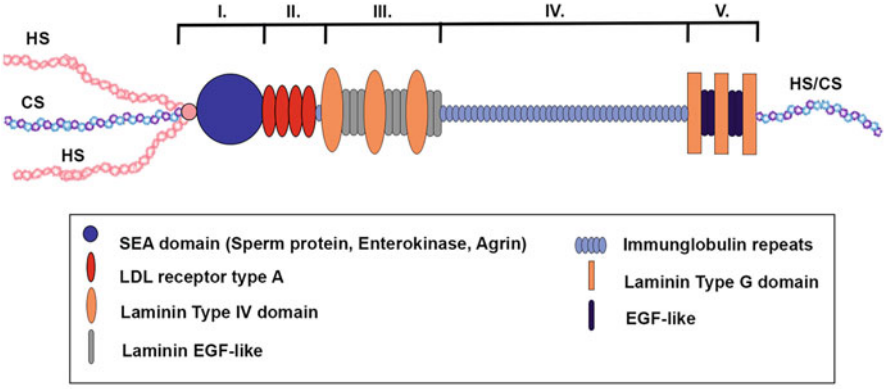
15.5.3 Heparin and Heparan Sulfate in the Regulation of Angiogenesis

Glycosaminoglycans (GAGs) play pivotal roles in the regulation of angiogenesis. GAG sugar chains are constructed of repeating glucuronic acid/*N*-acetyl glucosamine disaccharide units. This basic structure is modified by deacetylation and sulfation through the action of *N*-deacetylase/*N*-sulfotransferase and subsequently by epimerase that converts glucuronic acid to iduronic acid as well as three different *O*-sulfotransferases (Annaval et al. 2020). The ratio of *N*-sulfation to *N*-acetylation greatly determines the actual behavior of GAG chains, lending either stimulatory or inhibitory potential to the molecule. GAGs may exist as free sugar chains in the ECM, or may be attached to a core protein like syndecan and glypican on the cell surface or perlecan in the basement membrane (BM). Crucially to our topic, almost all factors involved in angiogenesis—close to 60 molecules we know of—can establish interactions with heparin or heparan sulfate (HS). Accordingly, GAGs may exert a wide variety of effects, the most prominent of which are the following:

(1) Heparin and HS may sequester angiogenic factors and thereby inhibit their binding to endothelial cells. (2) Proangiogenic growth factors may bind to the HS chains of perlecan and thus accumulate in the vicinity of endothelial cells. (3) HSPGs may present angiogenic signals to their respective cell surface receptors. (4) These signals, in turn, may facilitate the expression of cell surface HSPG or (5) Promote the synthesis of proteases. (6) Proteases may cleave HSPGs from the cell surface. (7) Free HS chains can bind to antiangiogenic factors and promote their effect (Chiodelli et al. 2015).

15.5.4 Discovery and Structure of Perlecan

Perlecan is a large proteoglycan that typically resides in BMs. Its protein core, sized over 470 kDa, is built up of five domains that show conspicuous homologies with other ECM proteins (Fig. 15.2) (Murdoch et al. 1992). The complete sequence of mouse perlecan was published in 1991 (Noonan et al. 1991), while the human sequence including the regulatory region was completed in 1993 (Cohen et al.



Interactive proteins with roles in matrix organisation and stabilisation.

Domain I.	Domain II.	Domain III.	Domain IV.	Domain V.
Laminin-1	Fibrillin-1	WARP	Nidogen-1	Nidogen-1
Collagen IV, V, VI, VII, XI, XII, XIV.	VLDL	Collagen VI	Nidogen-2	Fibulin-2
	LDL	Tropoelastin	Collagen IV, VI,	ECM-1
Col99, ColQ	Wnt		Fibulin-2	Collagen VI
Fibronectin			Tropoelastin	Fibulin-2
PRELP, WARP				Tropoelastin
Fibrillin-1, TSP-1				α-dystoglycan
Histone H1				matriglycan

Fig. 15.2 Domain structure of perlecan showing its most important interacting partners. Glycosaminoglycans binding critical regulators of angiogenesis like VEGF, FGF, and PDGF are attached to domain I of the molecule. In contrast to the proangiogenic action of perlecan, domain V, if liberated by protease activity renamed endorepellin, gains the potential to inhibit angiogenesis. *Int. J. Mol. Sci. 2021*, *22*(5), 2716

1993). Generally, perlecan carries HS GAG chains attached to the first domain, but its glycanation by chondroitin sulfate has also been reported (Lord et al. 2014). Domain I is also designated as SEA for its homology with sperm protein, enterokinase, and agrin. The other four domains (II-V) are built up from several modules and display homologies with LDL receptors, laminin, and the immunoglobulin superfamily (Farach-Carson et al. 2014; Melrose et al. 2006). This multidomain structure enables perlecan to establish interactions with a multitude of ECM molecules including BMs components like laminins and type IV collagen, interstitial matrix components like fibronectin, thrombospondin, and fibrillin, as well as growth factors, integrins, and others. (Bix and Iozzo 2008; McCarthy 2015; Mongiat et al. 2003a). This implies that besides its structural role perlecan possesses regulatory functions as well (Melrose 2020).

15.5.5 Perlecan Is an Indispensable Component of Basement Membranes

Type IV collagen and laminins are the principal building blocks of BMs that form a mat-like support for epithelial cells (Hohenester and Yurchenco 2013) or, as atypical BM, surround individual cells of mesenchymal origin (Pastor-Pareja 2020). Perlecan and nidogen are interspersed irregularly in this structure and connect collagen and laminin layers. Interactions of perlecan with other components of the BM both via its protein core and its HS chains adds stability to the structure. Besides acting as a molecular sieve, perlecan stores active molecules such as growth factors and cytokines, creates connections between growth factors and their cognate cell surface receptors, and promotes interactions between extracellular matrix molecules and their integrin receptors on the cell surface (Bix and Iozzo 2008; Gubbiotti et al. 2017). The importance of perlecan was clearly supported by the fact that its complete lack results in embryonic lethality in both mice and humans owing to inadequate vasculogenesis (Arikawa-Hirasawa et al. 1999; Iozzo et al. 2009).

15.5.6 Physiological Functions of Perlecan

Its large size and structural diversity endow perlecan with a plethora of physiological functions. As a component of the cartilaginous pericellular matrix it supports the interaction between chondrocytes (Wijeratne et al. 2016) and stimulates their proliferation in the growth plate via binding FGF 18; also, its domain I facilitates chondrogenic differentiation and supports the bone-forming activity of BMPII (Yang et al. 2006; Decarlo et al. 2012). Hence, perlecan is indispensable for proper skeletal development (Smith et al. 2007) and is a key component of all weight-bearing connective tissues including the articular *cartilage*, intervertebral disk,

meniscus, ligaments, and tendons (Guilak et al. 2021). Since perlecan is a prominent player of vasculogenesis and angiogenesis both in physiology and pathology (Bix and Iozzo 2008), its lack impairs cardiovascular development (Sasse et al. 2008) and hinders wound healing (Lord et al. 2017). Growth factors and cytokines attached to the HS chains of domain I can modulate cellular functions such as proliferation, migration, and angiogenesis (Lord et al. 2014). Perlecan regulates embryonic development by interacting with sonic hedgehog action (Palma et al. 2011). Via its domain V perlecan supports the function of pericytes in the maintenance and repair of the blood-brain barrier (Roberts et al. 2012; Nakamura et al. 2019). Conversely, the same perlecan domain V when disengaged by protease cleavage is called endorepellin and displays antiangiogenic properties (Mongiat et al. 2003b).

15.5.7 Perlecan in Pathology

Its modular structure and the great number of its interaction partners explain why perlecan also participates in so many pathological processes. The low-density lipoprotein (LDL) receptor-like domain II binds LDL and very low-density lipoprotein (VLDL), thereby interfering with lipid metabolism. This may impair muscle tissue through lipid deposition and facilitate the development of fatty liver, arteriosclerosis, and metabolic syndrome. Experiments utilizing perlecan-deficient mice rescued from perinatal lethality revealed that these animals have less and smaller white fat adipocytes. This cell type secretes adiponectin, a cytokine that prevents fat deposition into other organs (Yamashita et al. 2018). These results drew attention to a potentially unfavorable impact of perlecan on lipid metabolism (Vikramadithyan et al. 2004). Perlecan has also been shown to promote arteriosclerosis and osteophyte production in knee osteoarthritis (Kaneko et al. 2013). As a reservoir of cytokines and growth factors perlecan participates in wound healing by attracting fibroblasts and supporting the proliferation of capillaries. However, the same mechanisms can result in pathological fibrosis in chronic injuries or inflammation (Zhou et al. 2004).

In spite of these negative effects, perlecan is indispensable for embryonic development and tissue organization (Hassell et al. 2002). Various mutations of perlecan including missense, deletion, and splicing mutations may result in Silverman-Handmaker-type dyssegmental dysplasia (DDSH) (Hassell et al. 2002), skeletal dysplasias, and a combination of chondrodysplasia and myotonia characterized by disrupted neuromuscular junction called Schwartz-Jampel syndrome (Nicole et al. 2000; Arikawa-Hirasawa et al. 2002).

15.5.8 Perlecan and Cancer

Perlecan is thought to promote cancer progression by attracting ECM proteins that support tumor growth and via its HS chains that bind growth factors and facilitate

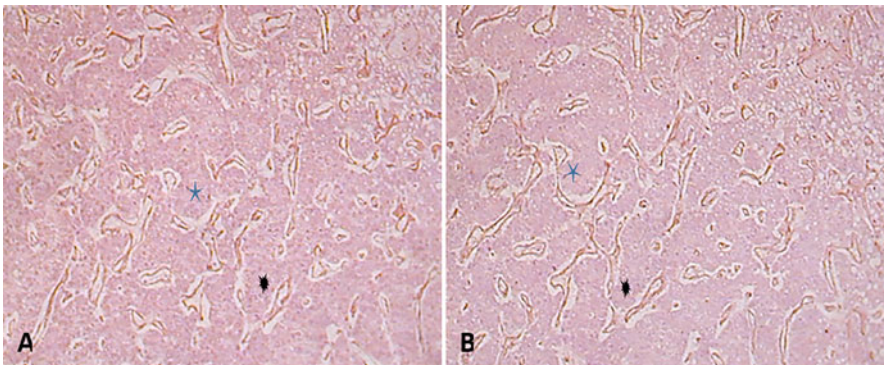


Fig. 15.3 Colocalization of perlecan and bFGF in the tumorous blood vessels of hepatocellular carcinoma. (**a**) perlecan, (**b**) bFGF. Original magnification x10. Asterisks show the regions positive for both perlecan and bFGF

their interaction with their cognate receptors on the surface of tumor cells. In experiments, p53-mutant pancreatic cancer cells could instruct their TAFs to upregulate the expression of perlecan and thus enhance the chemotherapy resistance and invasiveness of the tumor (Vennin et al. 2019; Ritchie et al. 2020) (Fig. 15.3).

In prostate cancer patients, increased serum levels of MMP7 and a tryptic fragment of perlecan domain IV are markers of invasiveness (Grindel et al. 2016); conversely, downregulation of perlecan reversed the invasive phenotype of prostate cancer (Savorè et al. 2005). Perlecan maintains SHH signaling in prostate cancer (Datta et al. 2006). Knockdown of agrin and perlecan inhibits the migration and adhesion of oral cancer cells and sensitizes them toward cisplatin (Correction: Agrin and perlecan mediate tumorigenic processes in oral squamous cell carcinoma 2015; Kawahara et al. 2014).

Besides supporting local spread, perlecan also promotes invasion and distant metastasis in multiple tumor types. The metastatic cascade is a multistep process that begins with the degradation of the basement membrane in the originating tissue and continues with migration through the extracellular matrix, intravasation into blood or lymphatic vessels and extravasation at a distant site where the tumor cells colonize. To successfully proceed through all these steps, tumor cells must permanently destroy epithelial and vascular BMs where they establish contacts with perlecan which in turn promotes colonization in the host tissue via its multivalent interactions (Elgundi et al. 2020). Upregulation of perlecan has been described in melanoma, lung, breast, liver, cholangiocellular, prostate, ovarian, pancreatic and oral carcinomas, as well as in glioblastoma (Cohen et al. 1994; Ilhan-Mutlu et al. 2016; Nackaerts et al. 1997; Nerlich et al. 1998; Hagedorn et al. 2001; Roskams et al. 1998; Sabit et al. 2001; Ida-Yonemochi et al. 2002; Kazanskaya et al. 2018; Warren et al. 2014; Sharma et al. 1998).

15.5.9 Perlecan and Tumor Angiogenesis

As perlecan is an indispensable resident of both epithelial and vascular BMs, its role in tumor angiogenesis comes as no surprise. Mechanistically, perlecan is thought to facilitate angiogenesis via its HS chains attached to the first, N-terminal domain. These negatively charged GAG chains bind several factors with angiogenic potential and present them to their receptors on the surface of endothelial cells. As discussed before, the best known and most potent of these factors is VEGFA that binds avidly to VEGFR 1 and 2 (Jiang and Couchman 2003); however, a host of other factors are induced by the hypoxic internal environment within fast-growing tumors. Basic FGF, PDGF, HGF, cytokines, and microRNAs—essentially the same factors detected in physiological angiogenesis—bind to perlecan, mostly to HS chains or occasionally to domain III, and stimulate endothelial cells to produce new blood vessels. What distinguishes tumor angiogenesis is dysregulation. Experimental evidence indicates that tumor-associated perlecan is not restricted into the BMs, but can also be detected in the tumor stroma, where it is synthesized both by stromal fibroblasts and tumor cells (Sabit et al. 2001). At the same time, deposition of perlecan diminishes in the fragmented basement membranes of the tumor tissue (Nackaerts et al. 1997). Tumor blood vessels fail to differentiate into arterial and venous arms, and their structure is abnormal with a decreased number of pericytes (Fig. 15.1) (Cao 2009).

15.5.10 Endorepellin, the Angiostatic Fragment of Perlecan

Similar to other basement membrane proteins like collagen type IV and XVIII, a fragment of perlecan can be shed via protease cleavage. Perlecan domain V, once released, is called endorepellin and it is known to inhibit angiogenesis and vasculogenesis by binding to VEGFR2 in regions different from the VEGFA binding sites. This interaction initiates signaling through VEGFR2 and α2β1-integrin that antagonizes the effect of angiogenic factors. The fact that endorepellin needs both VEGFR2 and α2β1-integrin receptors to exert its angiostatic effects confers specificity to its action, as only endothelial cells express both receptors on their surface (Goyal et al. 2011). Allosteric inhibition of VEGFR2 triggers internalization of the receptor, as well as downregulation of PI3K- and PLCγ-related signaling required for the transcription of VEGFA (Goyal et al. 2012). Although both angiostatin and endorepellin bind to VEGFR2, they utilize different integrins and different pathways: endostatin competes with VEGF for VEGFR2 and engages glypican/Wnt signaling and α5β1-integrin, whereas endorepellin binds to its own binding site on VEGFR2 and engages α2β1-integrin (Poluzzi et al. 2016).

15.5.11 Autophagy and Tumor Angiogenesis

Autophagy is a physiological process aimed to clear damaged, degraded, or hazardous molecules from the cytoplasm by delivering them to the lysosomes via autophagosomes. Autophagy helps to maintain intracellular homeostasis and assists adaptation to restricted nutrient supply (Schaaf et al. 2019). Thus, autophagy supports normal cells under starvation, prevents the accumulation of defective proteins, and participates in the rejuvenation of organelles (Oliva Trejo et al. 2020). Fast-proliferating malignant tumors with poor blood supply are even more dependent on autophagy for survival, as self-degradation provides tumor cells with nutrients under hypoxic conditions (Douglass et al. 2015; White 2012). On the other hand, when endorepellin binds to VEGFR2 on endothelial cells of tumor blood vessels it promotes not only autophagy but also cell death, which further potentiates its angiostatic effect (Poluzzi et al. 2014). Again, perlecan, and endorepellin have opposing roles as intact perlecan was shown to inhibit autophagy in muscles and thus maintain homeostasis (Ning et al. 2015; Gubbiotti et al. 2017). Of note, although perlecan and endorepellin have been studied most thoroughly in the context of tumor angiogenesis and autophagy, other ECM proteoglycans like decorin, biglycan, endostatin, and lumican have recently been found to display such activities (Chen and Iozzo 2020).

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Chapter 16
The microRNA-Extracellular Matrix Interplay in Breast Cancer

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Abstract The subclonal evolution of breast cancer is closely related to epigenetic regulation. Secreted microRNAs (miRNAs) spotted within the complex extracellular matrix (ECM) network are responsible for post-transcriptional and functional alterations to matrix constituents affecting vital cell processes for the initiation of metastasis, such as cell proliferation, migration, and invasion. The focus of this chapter is to highlight how the two-way relationship between miRNAs and ECM affects breast cancer pathogenesis and progression. Future investigation on the epigenetic regulation of matrix biomolecules focusing on miRNAs will improve current approaches to target tumor microenvironment and may expand our perspective in the mechanistic aspects of this pathology, contributing to a more effective breast cancer patient management.

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16.1 Introduction

MicroRNAs (miRNAs) hold an important role as post-transcriptional regulatory non-coding RNAs that target 3′ untranslated region (UTR) of one or multiple mRNAs (De Rie et al. 2017). Primary miRNAs are processed to precursor miRNAs (pre-miRNAs) by a specific microprocessor complex. Pre-miRNAs are then cleaved by the RNase III Dicer to generate mature miRNAs that direct the RNA-induced silencing complex (RISC) to mRNAs with complementary sequence (target-mRNAs) (Chendrimada et al. 2005).

MiRNAs are considered as powerful breast cancer biomarkers as they may govern several signaling cascades in (patho)physiological conditions including breast cancer, since more than 60% of protein-coding mRNAs may be targets of miRNAs as indicated by bioinformatics predictions (Cui et al. 2019). It is plausible to suggest that differential miRNA expression in a specific tissue or cell line affects the biogenesis and turnover of extracellular matrix (ECM), which constitutes a dynamic, multifunctional 3D network of macromolecules (Karamanos et al. 2021, 2018). Its constituents including proteoglycans (PGs), glycosaminoglycans (GAGs), collagen and elastin fibrils, glycoproteins, glycosidases and proteases, interconnect to form a regulatory niche that affect vital cancer cell functions, such as epithelial-to-mesenchymal transition (EMT), cell migration and invasion (Manou et al. 2020; Piperigkou et al. 2021). The miRNA-evoked control of the ECM has emerged as a novel mechanism of mediating matrix-dependent cancer cell processes, including cell proliferation, migration, adhesion, differentiation, apoptosis, and stem cell properties (Table 16.1) (Zolota et al. 2021; Piperigkou et al. 2018; Rutnam et al. 2013).

Opposing miRNA functions have been identified in breast cancer, as specific miRNAs may serve either as tumor suppressors or as oncogenes, depending on target-mRNA functions (Svoronos et al. 2016). MiR-21 and let-7 are among the most well-known breast cancer oncogenes and their plasma levels are crucial novel biomarkers for breast cancer monitoring (Khalighfard et al. 2018). Moreover, miR-155 is an oncogenic miRNA which has been mainly explored in leukemia (Ferrajoli et al. 2013); it also acts as an oncogene in breast cancer by targeting the suppressor of cytokine signaling 1 gene and promotes cell proliferation (Zhang et al. 2019). In breast carcinoma cells, miR-10b can directly suppress the translation (Ma et al. 2007). Moreover, the oncogenic axis of miR-10b involves the direct targeting of syndecan-1 to induce breast cancer cell migration and invasion (Piperigkou et al. 2016; Ibrahim et al. 2012). On the other hand, important tumor suppressors in breast cancer include miR-145(Wang et al. 2016), miR-205 (Chao et al. 2014), miR-335 (Heyn et al. 2011) and miR-372 (Zhao et al. 2017).

Targeting ECM and its cellular receptors through miRNAs is considered a novel mechanism to control matrix-dependent cellular processes that guide breast cancer progression. It is well established that miRNAs modulate the expression of matrix components, thus mediating cancer cell properties (Piperigkou and Karamanos 2019); however, there is a lack of evidence regarding the mechanistic aspects of

Table 16.1 MicroRNA-mediated regulation of extracellular matrix molecules in breast cancer

miRNA	ECM-related target	Cell line	Theranostic value	Reference
Breast cancer				
miR-10b	SDC-1	MDA-MB-231, MCF-7	– MiR-10b has a reported clinical correlation in preinvasive breast cancer – Emerging biomarker for BC diagnosis (Bertoli et al. 2015)	Ibrahim et al. (2012)
miR-140-3p	SDC-4	MDA-MB-231, SKBR3, MCF-7	Inverse relation between the impact of miR-140-3p and syndecan-4 on patient survival	Onyeisi et al. (2021)
miR-142-3p	ITGAV	MDA-MB-231, MDA-MB-468, MCF-7	Dysregulation of miR-142-3p in clinical samples of breast cancer and breast cancer cell lines compared to normal breast tissue	Schwickert et al. (2015)
miR-200b	LOX, SDC-2	MDA-MB-231, MCF-7	– Estrogen receptors mediate miR-200b expression in mammary cancer cells – Lower miR-200b levels have been correlated with poor disease-free survival (Piperigkou et al. 2020)	Sun et al. (2014)
miR-206	VEGF	MDA-MB-231, MCF-7	TNBC tissues express prominently lower levels of miR-206 compared to non-TNBC tissue samples and normal breast tissues	Liang et al. (2016)
ERα-positive				
miR-181a-5p	MMP14	MCF-7	miR-181a-5p is a critical regulator for MMP-14 expression and can affect MMP-14-mediated cancer cell migration, invasion, and angiogenesis	Li et al. (2015)
miR-221/222	ITGB4, ADAM17	MCF-7	– Integrin β4 expression was associated with poor primary luminal Lum-IC differentiation (G3 tumors) and with low miR-221/222 expression – An inverse correlation of miR-221/222 with Ki67 is also observed	Dentelli et al. (2014)
miR-373	CD44	MCF-7	Metastasis-promoting microRNA	Negrini and Calin (2008)
miR-520c	CD44	MCF-7	Metastasis-promoting microRNA	(Negrini and Calin (2008)

(continued)

Table 16.1 (continued)

miRNA	ECM-related target	Cell line	Theranostic value	Reference
ERα-negative				
miR-124	ITGB1	MDA-MB-231	MiR-124 is related to blockage of metastasis in ER- cell lines compared to ER+ cell line	Lv et al. (2011)
miR-1258	HPSE	SUM-149	MiR-1258 levels inversely correlate with metastatic ability of human breast cell lines and patient tissues	Zhang et al. (2011)
miR-143	CD44	SKBR3	MiR-143 functions as a tumor suppressor in breast cancer and that low miR-143 expression in breast cancer tissues may be an unfavorable prognostic factor	Yang et al. (2016)
miR-145	MMP11	HCC1937, MDA-MB-231	MiR-145 expression was downregulated in a graded manner in TNBC, with higher grades showing lowest expression	Tang et al. (2016)
miR-181a miR-193a/b	uPA	MDA-MB-231, MDA-MB-436	MiR-193a/b and miR-181a inhibit in vitro invasion of breast cancer cells mainly by downregulating uPA expression	Noh et al. (2011)
miR-193b	uPA	MDA-MB-231	MiR-193b is closely associated with clinical metastasis	Li et al. (2009)
miR-205	LamC1	MDA-MB-231, BT-549, SUM-149	MiR-205 is a novel transcriptional target of p53, and it exerts a role as oncosuppressor in triple-negative breast cancer, and in particular in a model representative of the most undifferentiated and mesenchymal subgroup	Piovan et al. (2012)
miR-205	VEGF-A	MDA-MB-231	MiR-205 is specifically downregulated in breast cancer	Wu et al. (2009)
miR-205	ITGA5	SUM-159	MiR-205 expression level is drastically lower in basal mesenchymal-like TNBC cells than other subtype breast cancer cells	Xiao et al. (2018)
miR-21	TIMP-3	MDA-MB-231	– Correlation of miR-21 with lymph node metastasis – Promising of therapy with	Song et al. (2010)

(continued)

Table 16.1 (continued)

miRNA	ECM-related target	Cell line	Theranostic value	Reference
			miRNA-based or non-miRNA treatments (Bertoli et al. 2015)	
miR-29b	ITGA6 (CD49f), LOX, VEGF-A	MDA-MB-231	MiR-29 expression correlates with more favorable outcomes, more differentiated phenotypes in normal and cancer cells and reduced metastatic potential	Chou et al. (2013)
miR-301	Col2A1	MDA-MB-231	Patients with higher miR-301 expression level have a worse DFS survival compared to those with lower miR-301 expression, while the relapses associated with miR-301 expression were nodal or distant metastases, not local recurrences	Shi et al. (2011)
miR-301a-3p from MDA-MB-231	TIMP-2 of astrocytes when they internalize EVs from BCC	MDA-MB-231	Higher levels of miR-301a-3p were significantly associated with reduced survival	Morad et al. (2020)
miR-30a-5p	ITGB3	MDA-MB-468, MDA-MB-231	– MiR-30a-5p expression strongly correlated with histological grade and survival status – High β3 integrin expression was markedly associated with reduced overall survival in TNBC patient subgroup	Li et al. (2016)
miR-31	ITGA2, ITGA5, ITGAV, ITGB3	MDA-MB-231	MiR-31 regulates cancer invasion-metastasis cascade	Augoff et al. (2011)
miR-335	TNC	MDA-MB-231	Expression of miR-126 and miR-335 is lost in the majority of primary breast tumors from patients who relapse, and the loss of expression of either microRNA is associated with poor distal metastasis-free survival TNC is involved in CSC immune evasion pathways	Tavazoie et al. (2008) and Graham et al. (2017)
miR-33a	ADAM9	MDA-MB-231	– MiR-33a expression is reduced in breast cancer tissues compared with matched normal tissues. – A correlation is observed between lower miR-33a	Zhang et al. (2015)

(continued)

Table 16.1 (continued)

miRNA	ECM-related target	Cell line	Theranostic value	Reference
			expression and increased lymph node metastasis	
miR-373	ITGA2	MDA-MB-468, MDA-MB-231	MiR-373^{high}/ITGA2^{low} may be a prognosis biomarker for breast cancer patients	Ding et al. (2015)
miR-4443	TIMP2	MDA-MB-231	MiR-4443 probably added to the chemoresistant capability of breast cancer cells by targeting TIMP2	Chen et al. (2016)
miR-506	CD151	MDA-MB-231	MiR-506 played a role as a master suppressor of EMT in breast cancer	Arora et al. (2013)
miR-520a-3p	CD44	Hs578T, BT20	MiR-520a-3p may play a suppressing role in breast cancer	Li et al. (2017)
miR-539	LamA4	BT-549	LAMA4 plays an important role in tumor progression and may be an important target in the treatment of TNBC	Yang et al. (2018)
miR-645	uPA	MDA-MB-231	uPA downregulation by miR-645 results in decrease of invasion in vitro and in vivo	Meng et al. (2018)
miR-9 from MDA-MB-468	ECM glycoprotein fibulin-3 EFEMP1 of normal fibroblasts	MDA-MB-468	– MiR-9 expression in TNBC correlates with different CAF subsets and resistance to cisplatin – EFEMP1 downmodulation is linked to the acquisition of a malignant phenotype in tumor-associated fibroblasts, which seems to be particularly relevant in TNBC subtype. – Emerging biomarker for BC diagnosis (Bertoli et al. 2015)	Cosentino et al. (2020)

Abbreviations: ADMA a disintegrin and metalloproteinase, EFEMP epidermal growth factor-containing fibulin-like extracellular matrix protein, HPSE heparanase, ITG integrin, Lam laminin, LOX lysyl oxidase, MMP matrix metalloproteinase, SDC syndecan, TIMP tissue inhibitor for matrix metalloproteinases, TNC tenascin C, uPA urokinase-type plasminogen activator, VEGF vascular endothelial growth factor

matrix-mediated miRNAs in breast cancer progression. Recent reports indicate that estrogen receptors (ERs) are responsible for the epigenetic targeting of miRNAs with established effects on breast cancer progression, such as miR-10b, miR-21, miR-145, and miR-200b in hormone- and non-hormone-dependent breast cancer cells (Piperigkou et al. 2017, 2020). In the aspect of matrix receptors, integrin β4

suppresses miR-92ab, miR-99ab, and miR-100, than in turn stimulate integrin β4-mediated cell motility in breast cancer (Gerson et al. 2012a). Moreover, circulating miRNAs loaded in tumor-derived exosomes or traveling within Argonaute (AGO) protein-positive ribonucleoprotein multivesicular bodies, functionally affect recipient cells, thus serving as critical biomarkers to control vital steps during breast cancer progression (Sun et al. 2018; Turchinovich et al. 2016).

This chapter focuses on the functional relationship among miRNAs and ECM biomolecules in the projection of breast cancer pathogenesis and development. Specifically, major examples of the well thought out effects of miRNAs on ECM components will be discussed. More focus is given to the functional relationship of matrix-mediated miRNAs in breast cancer, which is less elucidated.

16.2 ECM Regulates Breast Cancer Cell Progression Through miRNA Modulation

Altered miRNA expression in human cancers indicates that miRNAs contribute to tumor development (Chou and Werb 2012; Melo and Kalluri 2013). miRNAs are known regulators of matrix expression and through this pathway play an important role in cancer cell invasion and metastasis (Table 16.1). Recent reports indicate that ECM biomolecules have the ability to modify the expression and function of miRNAs (Fig. 16.1) (Bourguignon 2019). In the next sections, we review published data concerning the effect of ECM components on miRNA modulation in breast cancer cells. Investigating and understanding the mechanisms of interaction of the extracellular substance with miRNAs will allow the emergence of therapeutic targets related to these biological processes.

Most studies have focused on the effect of major ECM macromolecules, such as integrins, CD44, and hyaluronan (HA) on miRNA expression. An association between β4 expression and poor prognosis in "basal-like" breast cancer (Lu et al. 2008) has been proposed and the role of this integrin in breast cancer progression has been highlighted. First, Gerson et al. concluded that β4 integrin status affects the expression of specific families of miRNAs (Gerson et al. 2012a). In particular, through the performance of a novel miRNA microarray analysis (qNPA) they identified two miRNA families, miR-25/32/92abc/363/363-3p/367 and miR-99ab/100, that are downregulated by β4 integrin expression. Analysis of Affymetrix GeneChip data identified 54 common targets of miR-92ab and miR-99ab/100 within the subset of β4-regulated mRNAs, revealing several genes known to be key components of β4- regulated signaling cascades and effectors of cell motility. A novel function for the β4 integrin has been also reported in downregulating miR-29a expression which targets secreted protein acidic and rich in cysteine (SPARC), a glycoprotein that plays an important role in ECM remodeling and cell invasion. Thus, miR-29a repression by β4 integrin facilitated tumor invasion (Gerson et al. 2012b). The above results show that the integrin not only activates multiple

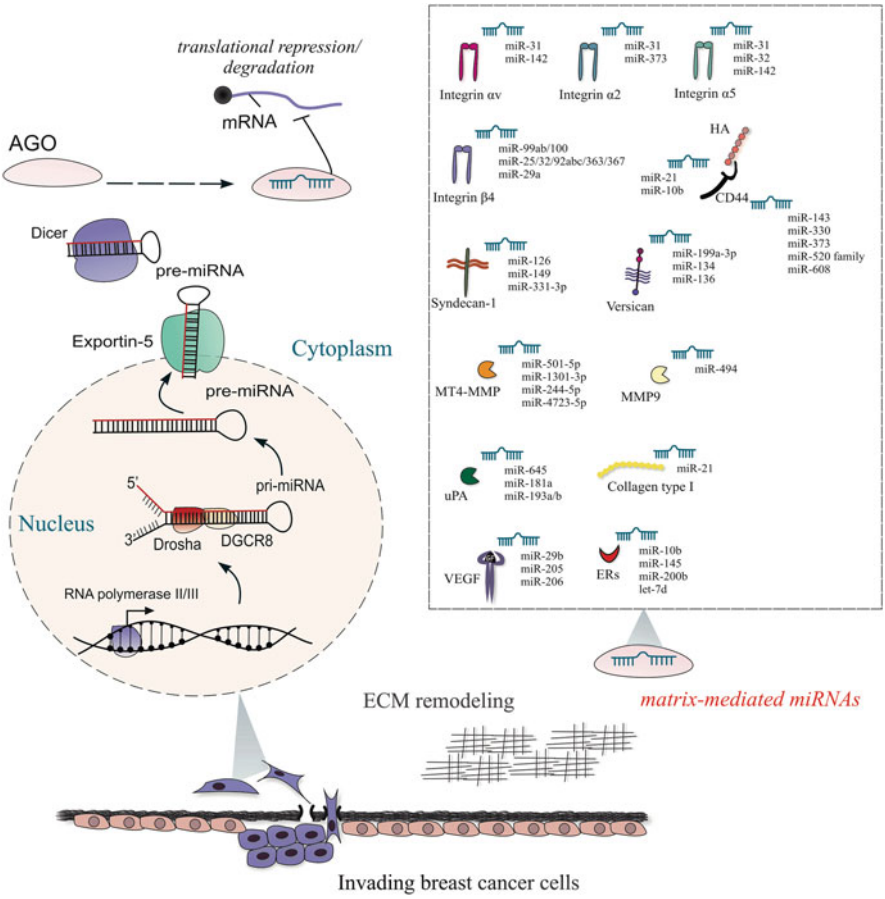

Fig. 16.1 MiRNA biogenesis and matrix regulation in breast cancer. The canonical miRNA biogenesis cascade produces mature miRNA sequences loaded to Argonaute (AGO) proteins to target the 3′ untranslated regions (UTR) of one or multiple mRNAs. Depending on target-mRNA functions, miRNAs act either as oncogenes or as tumor suppressors. During breast cancer progression, cancer cells-ECM affects the expression of certain miRNAs that in turn are involved in several steps of metastasis. Matrix-mediated miRNAs mediate cell-cell and cell-matrix interactions, thus regulating breast cancer cell behavior. Major ECM constituents, including integrins, estrogen receptors (ERs), syndecans, hyaluronan (HA)-CD44 interactions, matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), growth factor receptors (GFRs), and collagen type I, regulate the expression of several miRNAs that are involved in breast cancer progression

downstream signaling pathways through phosphorylation, but it also has the ability to regulate specific miRNAs which can influence β4-mediated migration and invasion.

HA and its binding receptor, CD44, have been studied in cancer and have been associated with tumor survival, proliferation, and migration (Tavianatou et al. 2021). It has been reported that HA-CD44 interaction exerts its carcinogenic effect through

miRNA signaling. Bourguignon et al. focused their research on detecting the mechanisms by which hyaluronic acid affects the expression miRNA in cancer and proposing novel drug targets for sensitizing tumor cell death. HA binding to CD44 promotes Nanog phosphorylation, a known stem cell marker, in the breast cancer cell line MCF-7. Phosphorylated Nanog through association with RNase III Drosha and RNA helicase p68 induces miR-21 production and program cell death 4 (PDCD4) reduction (Bourguignon et al. 2009). The final result of these events is inhibition of apoptosis, chemotherapy resistance, and blocking HA-CD44-mediated tumor cell behavior.

Moreover, it has been reported that HA binding to CD44 promotes c-Src kinase activation, which, in turn, increases Twist phosphorylation, leading to the nuclear translocation of Twist and transcriptional activation in MDA-MB-231 breast cancer cells (Bourguignon et al. 2010). Twist, which is one of c-Src substrates, is generally considered as a putative oncogene for its role in promoting a variety of tumor-related properties (e.g., EMT, invasion, and drug resistance). During HA/CD44 signaling, Twist induces miR-10b gene expression/production in tumor cells through interacting with miR-10 promoter (with E-box domain). This process results in the reduction of a tumor suppressor protein (HOXD10), RhoA/RhoC upregulation, Rho-kinase (ROK) activation, and breast tumor cell invasion. Src inhibitor, PP2, or Twist siRNA when applied to cancer cells significantly suspended miR-10 expression and downstream RhoGTPase (RhoC)-ROK effector functions (Bourguignon et al. 2010).

Therefore, it is suggested that the HA-CD44-mediated signaling events offer the potential for new therapeutic approaches that will target CD44 using anti-CD44 specific antibodies, inhibitors, anti-sense molecules, or HA-based nanoparticles containing therapeutic drugs.

With respect to the interaction with miRNA of additional single ECM macromolecules, recent data suggest that the metalloproteinase MT4-MMP (or MMP17) contributes in breast cancer growth and metastasis through regulation of miRNAs, including miR-501-5p, miR-1301-3p, miR-224-5p, and miR-4723-5p (Cervantes-Garduno et al. 2018). The targets of the most frequently altered (upregulated or downregulated) miRNAs affected several pathways that cooperate in hallmarks of cancer, including EMT and cell growth, and regulate the expression of transforming growth factor beta (TGF-β), mitogen activation protein kinase (MAPK), ErbB, and Wnt signaling, as well as several cell adhesion molecules.

ECM as a whole possesses biomechanical properties that affect cellular function (Manou et al. 2020). Composition and rigidity of ECM influences epithelial cell behavior and has recently been associated with miRNA regulation (Manou et al. 2020). Deposition of collagen type I promotes loss of epithelial polarity and tumor progression. Recently, studies using 3D cultures using Matrigel, which recapitulate the basement membrane matrix, explored a link between microRNAs and collagen type I in MCF-7 breast cancer cells (Li et al. 2011). Treatment of MCF-7 cells with collagen type I disrupted acini and upregulated the expression of miR-21, a well-documented oncogenic miRNA, via a post-transcriptional mechanism. The same group in two subsequent papers investigated the expression and function of miRNAs

in Matrigel 3D culture using tumor cells (Li et al. 2012; Nguyen et al. 2012). Comparing the expression of miRNAs in rBM 3D and 2D cultures of MCF-7 and MDA-MB231 cells, a profound difference has been revealed in miRNA profiles between the two cultures within each cell type, which also correlated with distinct mass morphogenesis. Collagen type I in rBM 3D culture substantially altered the miRNA signature of mass morphogenesis of MCF-7 cells. Overexpression of miR-200 family members, which are potent suppressors of EMT, in MCF-7 cells, correlated with strong cell-cell adhesion. In contrast, the silenced expression of miR-200 family members in MDA-MB-231 cells correlated with the stellate, mesenchymal, and highly invasive morphology (Piperigkou et al. 2017, 2020). Recently, the first miRNA signatures of morphogenesis of human breast cancer cells have been provided in rBM 3D culture and warrant further utilization of rBM 3-D culture in the investigation of miRNAs in breast cancer (Nguyen et al. 2012).

Using a similar approach identified significant differential expression of miRNAs between cancer cells cultured in Matrigel and those in 2D cultures (Price et al. 2012).

A common Matrigel-induced miRNA signature comprised of upregulated miR-1290 and miR-210 and downregulated miR-29b and miR-32 was identified using RT-qPCR across five epithelial cancer cell lines (SW480, SW620, HT-29, A549, and MDA-MB-231). Integrin α5 was identified as a novel putative target of miR-32 that may facilitate cancer cell interactions with the ECM. The authors proposed Matrigel culture as a valuable approach to the in vitro study of miRNAs.

All these results highlight that dysregulated ECM deposition quality may affect miRNA expression and 3D culture is a principal investigational approach in this field. It has been shown that the gene expression signature of 3D culture of breast cancer cells has great prognostic value for breast cancer patients (Martin et al. 2008). However, the molecular mechanism by which 3D culture affects miRNA expression has not yet been unravelled. Matrigel has a heterogeneous composition, and it should be important to establish whether miRNA expression signatures in 3D vs. 2D cultures are affected exclusively by the stiffness of Matrigel or also by its components.

All previous studies partially explain how ECM components interact with miRNA and promote tumor progression (Fig. 16.1). However, further research is necessary to highlight the mechanisms by which other ECM molecules regulate miRNA biogenesis.

It is a common knowledge that the 3′ UTR contains both binding sites for regulatory proteins as well as miRNAs. It has been proposed that the 3′ UTR of ECM molecule mRNAs, containing miRNA-binding sites, can regulate miRNA levels and function (Lee et al. 2010, 2011; Jeyapalan et al. 2011). Data suggest that 3′ UTRs of mRNAs act as 'endogenous decoys' for miRNAs and subsequently inhibit miRNA functions, by blocking target mRNA suppression (Salmena et al. 2011). This function has been extended to versican, CD44, and nephronectin mRNAs. Lee et al. showed that expression of versican 3′ UTR induced cell, tissue, and organ adhesion through competing miR-199a-3p levels and function (Lee et al. 2010). They demonstrated that the overexpression of versican 3′ UTR interacted with endogenous miR-199a-3p, deliberating versican and fibronectin mRNA for

translation. Overexpression of versican 3′ UTR also reduced breast cancer cell growth by decreasing miR-199a-3p, miR-134, and miR-136 levels and therefore restoring phosphatase and tensin homolog (PTEN) function (Lee et al. 2010).

Furthermore, research demonstrated that the 3′ UTR of CD44 interacts with miR-216a, miR-330, and miR-608 and inhibits tumor growth in a human breast cancer cell line, partly through modulation of angiogenesis (Jeyapalan et al. 2011). Moreover, it has been shown that the overexpression of the CD44 3′ UTR results in enhanced cell motility, invasion, and cell adhesion in human breast carcinoma cell line MDA-MB-231 cells, as well as metastasis in vivo. Using computational analysis, it has been indicated that miRNAs that interact with CD44 3′ UTR also have binding sites in other matrix-encoding mRNA 3′ UTRs, including collagen type I α1 repressed by miR-328 and fibronectin type 1 repressed by miR-512-3p, miR-491, and miR-671 (Rutnam and Yang 2012). Expression of CD44, collagen type I α1, and fibronectin type 1 was synergistically upregulated in vitro and in vivo upon transfection of the CD44 3′ UTR (Rutnam and Yang 2012).

Finally, it was shown that overexpression of the 3′ UTR of nephronectin through binding to miR-378 stimulates cell differentiation in the osteoblast progenitor cells MC3T3-E1. 3′ UTR-transfected cells had enhanced levels of β-catenin and GSK3 which contributed to cell differentiation, through minimizing EGFR and ERK phosphorylation (Lee et al. 2011; Kahai et al. 2010).

Evidence from recent research data suggests that a reciprocal and harmonious collaboration is taking place between miRNAs and the ECM that facilitates the balance of the cell with its environment.

As more and more mechanisms and actions come to light concerning ECM and miRNA interplay, a great challenge is to detect which constituents are most important for breast cancer cell survival and metastasis among them. A better understanding of the role and function of ECM-dependent regulation of specific miRNAs may lead to new treatment design with targeted therapies.

16.3 Conclusions and Perspectives

The miRNA biogenesis machinery is dysregulated in human cancer through complex mechanisms, such as abnormal transcriptional control of miRNA genes and defective epigenetic changes. Depending on the biological function of target mRNAs, miRNAs serve either as a novel class of oncogenes or tumor suppressors; thus, they directly orchestrate multifaceted signaling factors that in turn regulate breast cancer phenotype and progression. Focusing on the endogenous processing machinery by targeting the ECM-evoked miRNAs in breast cancer may help to better control cancer cell behavior that benefits patient management.

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Chapter 17
The Impact of the Extracellular Matrix on Immunotherapy Success

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Abstract Biological therapies to promote and enhance the immune response against cancer have gained much relevance in the past recent decade. Since the first approval of immune checkpoints inhibitor immunotherapies by the American Food and Drugs Administration (FDA), the outcomes obtained in patients with a variety of solid tumors have stimulated a revolution regarding this type of target therapy. Therefore, therapeutic options and developments aimed at enhancing the immune response in patients are currently increasing. The evasion mechanisms mediated both by tumors and by the tumor microenvironment (TME), however, remain important barriers to acquiring a satisfactory antitumor response. The dynamic extracellular matrix (ECM) as a part of the TME plays a key role in this scenario. In this chapter, we addressed how the interplay between cancer cells and the TME components, including soluble factors, endothelial cells, tumor-associated macrophages (TAMs), T cells, cancer-associated fibroblasts, and a perturbed ECM, could contribute to cancer progression. Importantly, how the TME remodeling, especially ECM changes, are implicated in the success of cancer immunotherapy.

17.1 Introduction

Conventional strategies for advanced cancer patients with unresectable tumor include chemotherapy and radiation. Developing novel strategies such as antiangiogenic or immunotherapy, on the other hand, consists of targeted therapies. Even with the wide range of therapeutic options, the improvement of clinical response and/or survival of patients remains limited. Currently, there is a vast experience regarding the application of immunotherapy in tumors, and it is widely known that immune cells are involved in the control of cancer. During the last two

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decades, a growing area of research focused on the novel strategies, acting in many phases of the immune response regulating the innate and the adaptive immunity. In addition, many studies indicate that conventional chemotherapy and/or radiotherapy could act in synergy to generate immunity against many tumors. However, many immune evasion mechanisms and events, such as cancer cells' uncontrolled growth, resistance to apoptosis, or metabolic shift toward anaerobic glycolysis, trigger an adjustment of the extracellular matrix (ECM), modifying the response from stromal and immune cells and impacting on the efficacy of immunotherapies. Then, ECM composition and dysregulation become key drivers for both cancer progression and response to antitumor therapies. In this chapter, we will discuss how the ECM remodeling influences the immune response against cancer and how it can be harnessed to achieve the benefit of immunotherapy for eradicating tumors.

17.2 Extracellular Matrix Remodeling and Immune Tumor Microenvironment

17.2.1 ECM Components

The ECM is a dynamic network of macromolecules such as fibrillary collagen, elastin, glycosaminoglycans (GAGs), proteoglycans (PGs), and enzymes with essential support and biochemical function for cells and tissues. These extracellular macromolecules, produced mostly by fibroblast, are in constant and highly controlled remodeling. Abnormal changes or dysregulation in the conformation of ECM components could alter chemical and physical ECM properties and promote tumor progression (Bonnans et al. 2014).

1. *Fibroblasts and Cancer-associated Fibroblasts (CAFs):* Fibroblasts synthesize collagen and other fibrillar and non-fibrillar factors maintaining the ECM homeostasis. They produce the network to support tissue structures and integrity. Fibroblasts can acquire an immune regulatory phenotype and in cancer could play an important role as predominant cell type from the tumor stroma (Pankova et al. 2016). This subpopulation widely identified as cancer-associated fibroblasts (CAFs) are present in many types of solid tumors. CAFs are recruited from resident fibroblasts, bone marrow-derived progenitor cells, or by cancer cells (McDonald et al. 2015). They are activated in the TME, by factors such as the transforming growth factor-beta (TGF-β), secrete by tumor to become myofibroblasts (Calon et al. 2014). In addition to cancer cells, inflammatory and immune cells also can produce mediators to activate fibroblasts. CAF interactions within tumors are complex, and it is depending on specific tumor types and the cells of their TME but generally, CAFs and TME cells interrelate contributing to tumor growth (Takahashi et al. 2017). For instance CAFs isolated from breast cancer triple-negative tumors expressed the monocyte attractant chemokine CXCL16, stimulated by myeloid suppressor cells to, in turns, recruits

more myeloid cells and more fibroblasts (Allaoui et al. 2016). Myeloid-derived suppressor cells (MDSCs), as we will describe later, are an immunosuppressive population that impairs T-cell response. MDSCs and CAFs accumulation then promotes stroma activation, thickening of ECM, immune evasion, and cell metastasis, and is associated with poor prognosis in this aggressive phenotype of breast tumor (Takai et al. 2016). The accumulation of CAFs was also associated with the numbers of CD163-positive tumor-associated macrophages (TAMs) in oral squamous cell carcinoma (OSCC). For instance CAFs release IL6, CXCL8, and TGF-β determining M2 TAM polarization, influencing the TME to an immunosuppressive milieu and inducing protumoral phenotype of TAMs in OSCC (Takahashi et al. 2017).

2. *Collagens:* Type I fibrillar collagen as well as other types of collagens are secreted by fibroblasts to form macromolecules by cross-linking, conforming the most percentage of biomolecules of the ECM (Netti et al. 2000). The cross-linked collagen provides ECM flexibility but also complexity. On the one hand, type I collagen acts as a regulator of matrix stiffening, but also is implicated in growth factors and cytokines bioavailability across the ECM, impairing tumor immunity (Netti et al. 2000). It has been reported that aged fibroblast-derived collagen promoted biophysical changes in the ECM, inhibiting the motility of T cells and promoting the accumulation of myeloid cells, which results in an overall change in the immune profile through the ECM (Kaur et al. 2019). These events could have implications, for example, in the efficacy of current therapies for melanoma. In human breast cancer, collagen deposition and stiffness of ECM correlate with tumor invasion. In addition, it was observed that the number of infiltrating TGF-β-producing TAMs was highest in more aggressive tumor subtypes (Maller et al. 2021). Also, TGF-β secreted by TAMs activates collagen crosslinking, which is implicated, as we mentioned, in elasticity and complexity of the ECM and finally, in immune cells trafficking.
3. *Glycosaminoglycans:* the most abundant glycosaminoglycan (GAG) in the ECM is hyaluronan (HA) (Chanmee et al. 2016). HA is a high-molecular-weight GAG, synthesized by cell membrane hyaluronan synthases 1, 2, and 3 (HAS1–3), by alternative addition of glucuronic acid and N-acetylglucosamine. HA is involved in the elasticity and viscosity of the ECM because acts buffering ion exchanges, water, and also maintaining the osmotic balance. Then, HA also plays a key role in cell mobility, invasion, proliferation, and inflammation (Chanmee et al. 2016; Nikitovic et al. 2015).

   Heparan-, chondroitin-, and keratin-sulfate are other GAGs with viscoelastic effects on ECM. The addition of a sulfated site in this group allows GAGs to bind to proteins by covalent bonds. For example, chondroitin sulfate can bind to matrix proteins, soluble growth factors, chemokines, and cytokines interacting with water and then modulating ECM viscoelasticity (Berdiaki et al. 2021).
4. *Proteoglycans:* Proteoglycans (PGs) include a core protein with one or more covalently attached GAG side chains (Tzanakakis et al. 2019). PGs are important regulators in our body. Depending on their types they localize on the cell surface, in the ECM, and some of them is detected intracellular. Owing to their GAG

chains they are implicated in several biological and pathological events, including inflammation and cancer. Biglycan is the residence of the ECM, with diverse functions. Among those it is highly implicated in the development of inflammatory cancer milieu (Tzanakakis et al. 2019; Yamauchi et al. 2018). Secreted by the macrophages, it can stimulate their TRL2 and 4 receptors ending up by increased cytokine (TNFalfa, CCL2, CCL5, IL1beta, IFNgamma, CXCL13, etc.) synthesis. Its action concluding in the recruitment of neutrophils, macrophages, B and T lymphocytes promoting anti-apoptosis and tumor growth (Tzanakakais et al. 2019). Versican, another type of ECM PG, binds molecules such as GAGs (particularly HA) (Wight et al. 2020), the inflammation-associated protein tumor necrosis factor-stimulated gene-6 (TSG-6), and is also involved in leukocytes infiltration and cancer progression. In the TME, Versican interacts with cells both via HA or via receptors such as toll-like receptors (TLRs) or CD44 present on immune and non-immune cells. Versican is also involved in the availability of cytokines such as tumor necrosis factor alpha (TNFα), IL-6, and other soluble factors influencing on the phenotype of the immune cells in the TME (Wight et al. 2020). In this sense, it has been reported that Versican produced by CAFs lead to a pro-inflammatory milieu, while Versican expressed by MDSCs can lead to an anti-inflammatory and immunosuppressive TME (Hope et al. 2016).

5. *Matrikines and MMPs:* Matrikines are fragments produced from ECM macromolecules, i.e., collagen, elastin, and Versican partial degradation (Wells et al. 2015). Matrikines are also able to regulate many cellular processes such as proliferation and migration and have been described as regulators of physiological or pathological processes such as tissue repair, inflammation, and tumor invasion (Wells et al. 2015). Recently, it has been reported that Versikine—a fragment derived from Versican proteolysis—promotes CD8+ T-cell infiltration and generation of CD103 + $CD11c^{hi}MHCII^{hi}$ conventional dendritic cells (DCs), a population of antigen-presenting cells implicated in T-cell activation in colorectal cancer (CRC) (Hope et al. 2017). On the other hand, matrix metalloproteinases (MMPs) are a family of 28 zinc-dependent endopeptidases that principally contribute to fibrillar and non-fibrillar collagens degradation. Remodeling of ECM by MMPs supports tumor dissemination and invasion (Wells et al. 2015). In this sense, it has been demonstrated that adhesion molecules CD44 and integrin alphavbeta3 enable that activated MMPs binds on the cell surface of melanoma cells promoting ECM degradation, tumor growth, and cell invasion (Yu and Stamenkovic 1999).

17.2.2 Immunomodulatory Roles of ECM: Matrix Components as Promoters of Immune Response

Despite ECM usually being considered as a passive architectural structure, the ECM is now being accepted as a dynamic red, source of damage-associated molecular patterns (DAMPs) with the ability to activate the immune response. Those DAMPs

could be, for example, matrikines mentioned above (Shay et al. 2015). In homeostasis, the composition of the ECM is continuously adjusted by a sequence of synthesis and degradation events. Instead, during cancer progression, this dynamic balance could be disrupted and lead to the accumulation of ECM components and chronic inflammation. ECM deposition and transformation during tumorigenesis can result in the release or formation of ECM-derived DAMPs which can stimulate local inflammatory response and immune cell recruitment. ECM-derived DAMPs are capable of modulating immunity both directly via interaction with immune receptors such as CD44 and TLRs or indirectly through the release of cytokines and chemokines. Cells from innate and adaptive immune systems attracted by these soluble factors can be found within the ECM and the same immune cell type may delay or stimulate tumor progression. For example, the bioactive fragment Versikine, generated from Versican proteolysis by a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs (ADAMTS) protease, promotes immunogenicity and antagonizes DCs dysfunction promoted by whole Versican. In addition, Versikine promotes T-cell infiltration through regulation of a particular DC subset that is critical for CD8+ T-cell trafficking and antitumor immunity (Hope et al. 2017). On the other hand, matrikine Val-Gly-Val-Ala-Pro-Gly (VGVAPG), an elastin peptide, specially attracts monocytes to the TME while impairing neutrophil reactivity (Senior et al. 1984). Other elastin fragments are also released to the ECM during tumor progression. For instance, elastin-derived nonapeptide AG-9 stimulates OSCC cells' invasive properties by increasing membrane type 1-matrix metalloproteinase (MT1-MMP, also known as MMP-14 expression and MMP-2 secretion (Bretaudeau et al. 2020). Overexpression of MMP-14 in the TME could become critical since it has been reported that MMP-14 modulates inflammatory signaling networks for controlling macrophage-mediated immune response. Additionally, upregulated MMP14 levels correlated with a lack of CD8+ cytotoxic T cells in CRC (Claesson-Welsh 2020). In this manner, ECM can directly contribute to recruit specific immune cells but also to the lack of infiltrating antitumor immune populations in the TME.

17.3 Immune Response in Tumors

17.3.1 The Cancer-Immunity Cycle

Immune -mediate response to cancer is a cyclic process that can be self-propagating, primary to an increase of immune-stimulatory factors such as genetic and cellular alterations presents in cancer cells (Chen and Mellman 2013). These characteristics that define tumors provide the immune system with the resources to generate T-cell responses to recognize and eradicate malignant cells. This cycle can be distributed into many steps, from the release of tumor cell antigens to the cancer cells' elimination. First, neoantigens generated by tumorigenesis are released and taken by antigen-presenting DCs. In this step, many signals are crucial to achieve a suitable T-cell-mediated immunity. These signals might include cytokines secretion and

DAMPs released by dying tumor cells. Then, DCs present the captured antigens on class I major histocompatibility complex (MHCI) and class II MHC (MHCII) to the priming and activation of effector T cells. Next, CD4+ and CD8+ T cells leave secondary lymphoid organs adjacent to the tumor and return to tumor tissue to support a response against the cancer-specific antigens. The landscape of the immune response is determined at this step, with an important balance between CD4+ and CD8+ T effector cells versus principally CD4+ but also CD8+ T regulatory cells. Elimination of the cancer cell at the tumor site releases additional antigens, increasing the scale and complexity of the response in successive reiterations of the cycle. In addition, this sequence is characterized by inhibitory factors that produce regulatory mechanisms, which can restrict immunity. The presence of an immunosuppressive milieu may explain the partial efficacy observed in immune-based cancer therapies and why these therapies may be more active in combination with agents that goal other steps of the cycle (Chen and Mellman 2013).

17.3.2 Immune-Cell Trafficking in the Stroma

1. *Tumor-infiltrating regulatory T cells:* naturally occurring regulatory T cells (Tregs) are originated in the thymus as a subtype of $CD4^+$ T cells that constitutively express the CD25 molecule and the transcription factor forkhead box P3 (Foxp3) which is essential to suppress $CD4^+$ and $CD8^+$ T cells effector responses in vivo (Sakaguchi 2004). A sub-population has been also identified as *induced* Tregs, characterized by their ability to inhibit the effector T-cell response by the secretion of soluble factors. Tregs inhibit tumor-infiltrating lymphocytes (TILs) through immunosuppressive cytokines such as interleukin 10 (IL-10) and tumor growth factor beta (TGFβ) and by the surface expression of cytotoxic T lymphocyte antigen 4 (CTLA-4). In addition, Tregs block anti-tumor immunity impairing NK cells cytokine production, inducing tolerant DCs, and increasing the activity of 2,3 indoleamine oxygenase (IDO) which is responsible for tryptophan degradation resulting in $CD4^+$ and $CD8^+$ T cells apoptosis (Thompson and Powrie 2004)

   It has been reported that patients with lung, skin, breast, ovarian, and pancreatic cancers showed an increase in the number of $CD4^+$ $CD25^+$ $Foxp3^+$ cells. Currently, monoclonal antibodies (mAbs) directed against CTLA-4 are used in patients. However, systemic depletion of Tregs by checkpoint inhibition may induce autoimmune responses (Tanaka and Sakaguchi 2017). With the aim to induce antitumor immunity without the induction of autoimmunity is possible to target other cell type that enables immune evasion in the TME such as CAFs. For instance, recently it has been observed that CD70-positive CAFs significantly increased the frequency of Tregs in CRC. Then, consideration of CD70-targeting antibodies to revert the lack of anti-tumor immune response promoted by Tregs could be an interesting approach for CRC (Jacobs et al. 2018).

2. *Tumor-infiltrating Regulatory Myeloid Cells:* Myeloid-derived suppressor cells (MDSCs) are immature cells with immunosuppressive capability (Gabrilovich and Nagaraj 2009). In humans, MDSCs are characterized by $CD11b^{+}$ $CD14^{-}$ $HLA\text{-}DR^{-/low}$ $CD33^{+}$ $CD15^{+}$ markers, and their presence in cancer patients has been associated with poor outcomes. MDSCs can promote T cells anergy. In addition, MDSCs express the inhibitory ligand, PD-L1, causing an exhausted phenotype of effector T cells (Noman et al. 2014). Additionally, MDSCs can release nitric oxide (NO) and peroxynitrite inhibiting T-cell activation, induce the increase of Tregs, and differentiate macrophages to a M2 phenotype. Modulation of the number of MDSCs and or inhibition of MDSCs activity are currently areas of great interest (Deng et al. 2017).
3. *Tumor-infiltrating Cytotoxic T Lymphocytes (TILs):* The success of an adequate antitumor immune response depends on preceding recruitment of tumor-infiltrating CD8+ cytotoxic T lymphocytes (TILs) in the TME (Chen and Mellman 2013). Chemokines of CCL and CXCL family have been related to TILs enrollment in many solid tumors (Harlin et al. 2009). The frequency of TILs is a favorable prognostic in many cancers such as melanoma, esophageal, breast, ovarian, head and neck, and non-small-cell lung cancer (NSCLC). Diverse mechanisms, as direct tumor cell killing, have been described for the antitumor activity exerted by TILs (Boon et al. 1994).

   ECM dynamic changes and remodeling are critical for immune cell trafficking, migration, spatial distribution, and activation. DCs and T cells are able to migrate along type 1 collagen self-regulating adhesion molecules whereas cancer cells use MMPs and integrin-dependent migration to penetrate collagen fibrils. It has been observed that in lung cancer, T-cell infiltration arises in low-density fibronectin and collagen regions while it is decreased in dense matrix fibers.

17.3.3 The Role of the Matrisome in Tumor Inflammation and Cancer

The set of proteins with different functions from cellular adhesion and motility to cell signaling that conformed the ECM has been identified as the *matrisome* (Socovich and Naba 2019; Yuzhalin et al. 2018). Modifications in the matrisome because of tumor progression could cause reciprocal alterations in the cancer cells and activate pathways responsible for many hallmarks of cancer such as inhibition of apoptosis and uncontrolled proliferation. Recently, Yuzhalin et al. have identified a nine-gene matrisome signature common to a range of solid cancers, which predicts poor prognosis in several tumor types. Matrisome gene signatures from CRC, gastric, lung, and ovarian cancers were significantly enriched in hypoxia, neovascularization, epithelial-mesenchymal transition, and inflammation (Yuzhalin et al. 2018).

17.4 Cancer Immunotherapy

17.4.1 Improving Immune-Mediated Antitumor Response

Immunotherapy purposes control the growth and spreading of malignant cells by the activation of a specific immune response (Rosenberg et al. 2004). In this sense, a vast number of strategies to achieve an effective immune response against tumors have been tested such as cytokine or immune checkpoint blockade mAbs administration, adoptive T-cell therapy, dendritic cells-based vaccines, etc. Some of them are under evaluation and actually practice in the clinic, particularly the use of immune checkpoint inhibitors (Perez-Gracia et al. 2014).

These strategies have been demonstrated to be potent in animal models, but it was not until a few years ago with the use of a DCs-based vaccine in hormone-refractory prostate cancer (*sipuleucel*) or immunostimulatory mAbs (*ipilimumab, nivolumab, pembrolizumab, tremelimumab*) that clinical results were more satisfactory (Hodi et al. 2010). An elucidation for the unsatisfying clinical results is based on immunosuppressive mechanisms used by tumors cells to escape from the host immune response. This has led to the design of new approaches to inhibit immunosuppressive factors derived from the TME, in part responsible for the lack of activation of an efficient antitumor response.

In the design of a therapeutic strategy, the need to implement multiple approaches to block immune suppressor mechanisms must be taken into account. In this context, protocols of combined therapy with focusing on modulate matrix and TME components might act in synergy.

17.4.2 Immune Checkpoint Inhibitors

The immune response is regulated by a highly complex equilibrium of signals transmitted by stimulatory and inhibitory receptors (Tanaka and Sakaguchi 2017). Immunostimulatory monoclonal antibodies (mAbs) have the ability to enhance ongoing immune responses. A number of resistant metastatic cancers (e.g., melanoma, renal cell carcinoma, or lung cancer) now have the possibility of being successfully controlled using mAbs with long-term clinical responses (Tanaka and Sakaguchi 2017). The main concept involves the block of negative regulatory cell surface molecules, which inhibit T-cell activation. Immune checkpoint inhibitors (ICI) antibody therapy has demonstrated that the CTLA-4 inhibitor *(ipilimumab*, *tremelimumab*) has favorable antitumor activity in patients with advanced solid tumors (Hodi et al. 2010). Lately, fully human (IgG4) monoclonal anti-PD-1 (*nivolumab*, *pembrolizumab*) and programmed death receptor ligand-1 (PD-L1, *atezolizumab,* durvalumab) antibodies have demonstrated clinical benefit in melanoma, refractory non-small cell lung cancer, advanced renal cell carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, urothelial carcinoma and

hepatocellular carcinoma (Alsaab et al. 2017). Remarkably, anti-PD-1 therapy demonstrated longstanding survival, robust responses, and safety profiles in these tumor types. Moreover, for many types of solid tumors, clinical responses occurred regardless of PD-L1 expression on tumor cells. Re-establishing anti-tumor immunity seems to be feasible and the results with ICI are favorable; however, the efficacy of strategies based on immunostimulatory mAbs is vulnerable to the hostile TME that suppresses the effector response (Alsaab et al. 2017). In this sense, the role of ECM components might reveal potential future synergism for cancer treatment.

17.4.3 ECM Components as Emerging Players in Immunotherapy

As we mentioned, a successfully immune response rests on a tight balance between stimulatory factors and inhibitory factors. Regardless of immunotherapy with ICIs block checkpoints to induce anti-tumor response, stromal cells, including CAFs, ECM macromolecules (collagens, GAGs, and PGs), matrikines and cytokines are involved in the delivery and polarization of immune cells and regulates generation and proliferation of the sustained host immune response (Fig. 17.1a). Particularly, CAFs that secrete matrix-associated proteins, cytokines, and pro-inflammatory chemokines orchestrate the access of leukocytes in the TME (Freeman and Mielgo 2020). It has been reported that CAFs correlate with an immune-suppressive phenotype, with decreased IFN-gamma and granzyme B expression, supporting resistance to ICI therapy in experimental CRC models. In addition, CAFs derived from melanoma biopsies showed an upregulation of both programmed death ligands 1 and 2 (PD-L1 and PD-L2), abolishing CD8+ T-cell function (Li et al. 2019). PD-L1 and PD-L2 have also been upregulated in CAFs from pancreatic cancer patients. As well, CAFs promote the expression of other co-inhibitory immune checkpoint receptors such as LAG-3 and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) impairing effector T-cell function (Goehrig et al. 2019). It has been proposed that the upregulation of inhibitory checkpoints is mediated by secretion of soluble factors such as CXCL5, which enhanced the expression of PD-L1 in melanoma and CRC cell lines by activating PI3K-AKT pathway (Li et al. 2019). HCC-associated fibroblasts also were capable to upregulate the expression of PD-L1 on the surface of neutrophils, by IL-6 secretion. Then, neutrophils were then able to suppress T-cell activation in this HCC model (Cheng et al. 2016).

On the other hand, as we describe, matrikines are formed from collagen, laminin, elastin, Versican, and HA by MMPs and a family of matrix enzymes that include a-disintegrin-and-metalloproteinases (ADAM) and ADAMTS (Wight et al. 2020). Matrikines can directly contribute to recruit specific immune cells. To potentiate the efficacy of ICI, matrikines could be proposed to regulate T-cell infiltration and the availability of interleukins, promoting TME inflammation and contributing to

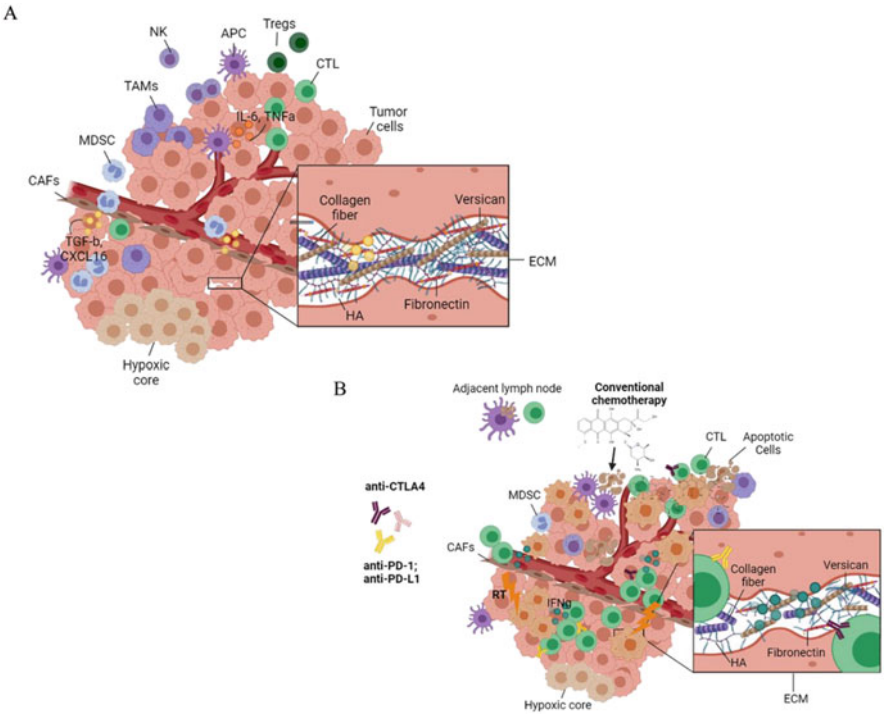

Fig. 17.1 The extracellular matrix plays a key role in the activation of TME immune response. (**a**) The dynamic network of stromal cells and macromolecules in constant remodeling supports the infiltration and polarization of immune cells. Fibroblasts and cancer-associated fibroblasts (CAFs) release soluble factors and matrix proteases degrade ECM proteins to produce matrikines (versikine, elastin-derived peptides) regulating the host immune response. Antigen-presenting cells (APC) such as dendritic cells (DCs) and T cells (cytotoxic T lymphocytes; CTL) migrate along collagen and fibronectin fibrils. ECM also is involved in the efficacy of immunotherapy: whereas matrikines can regulate T-cell infiltration and the availability of interleukins contributing to overcome resistance, hypoxia in the TME can promote immune evasion by upregulation of immunosuppressive factors (transforming growth factor-beta; TGF-b, CXCL16) and the recruiting of myeloid-derived suppressor cells and regulatory T cells (MDSCs; Tregs). (**b**) In spite of the demonstrated efficacy of immune checkpoint inhibitors (ICI) in advanced cancers, local radiotherapy (RT) and/or conventional chemotherapy can transform tumors and overcome some of the evasion mechanism. RT can induce an inflammatory response in the TME mediated by IL-1 and IL-6, the release of neoantigens, DCs presentation and T CD8+ cell activation with Interferon gamma (INF-g) secretion. In addition, chemotherapy could lead to tumor cell death by apoptotic and/or non-apoptotic mechanisms impacting on the cross-presentation of tumor-derived antigen mediated by DCs. Then, conventional chemotherapy and RT could promote immunity against many tumors and improve the efficacy of immunotherapy with immune checkpoint inhibitors such as anti-CTLA4, anti-PD-1, or anti-PD-L1

overcome resistance that suppresses the effector immune response pursued with immunotherapy.

17.5 Therapeutic Combinations to Enhance Immunotherapy Achievement

17.5.1 Influence of Radiotherapy on ECM and the Stroma

In spite of the demonstrated efficacy of ICI in advanced cancers, many patients do not experience therapeutic benefit, maybe by a lack of specific recognition, or to the presence of immunosuppressive mechanisms in the TME (Pitt et al. 2016). Recent advances have discovered that local radiotherapy (RT) can transform tumors and overcome some of the evasion mechanisms reported (Menon et al. 2019).

RT is a key tool to treat patients with cancer. Its main way of action is associated with its capacity to induce double strain DNA damage (DSD) triggering cell apoptosis. RT technics has been changing in parallel with technologies development. Hyper-fractionated RT has been classically used but, nowadays, there are a lot of different technics which administrate large doses of radiotherapy in few fractions.

These kinds of treatment could impact ECM affecting their cellular compartments like CAFs, MDSCs, and TAMs, but also induces changes in ECM density, composition, and stiffness likely modifying malignant cell invasion, survival, and proliferation (Menon et al. 2019).

In this sense, it has been shown that RT induces changes in CAFs modulating their genomic expression and regulating their pro-tumorigenic capability. They induce radioresistance by secreting CXCL1 and it was associated with worse prognostic in patients receiving RT (Zhang et al. 2017); in addition, it has been observed that STAT3 phosphorylation induced by RT promotes MDSCs infiltration and proliferation (Grinde et al. 2017; Allaoui et al. 2016; Oweida et al. 2021). However, the inhibition of this path in association with RT improves tumor response in pancreatic cancer (Oweida et al. 2021).

Thence, to maximize the therapeutic benefit, it is important to define the doses and also combinations to highlighting the pathways to promote anti-tumor immunity and effector T cell function while limiting pathways that mediate an immunosuppressive TME.

It has been reported that RT has the ability to generate immunogenic signals and release DAMPs (Bernier et al. 2004; Wu et al. 2017). In this context, RT is able to induce an inflammatory response in the TME mediated by cytokines such as IL-1 and IL-6. In addition, the release of DAMPs and tumor neoantigens induced by RT allows DCs presentation and T CD8+ cell activation (Garnett et al. 2004; Burnette et al. 2011).

RT also has been shown to promote the stimulator of interferon genes (STING) pathway activation and PD-L1 upregulation. When RT induces DNA damage and

tumor cell apoptosis, DNA from dying tumor cells is uptake by DCs. This event induces the sensing of DNA by cyclic-GMP-AMP with activation of STING pathway that finally induces expression of Type I IFNs. Additionally, in response to RT-induced tissue damage, CAFs can secrete chemoattractant molecules such as CXCL9 and CXCL10, recruiting CD8+ T cells in the TME (Chen et al. 2016) (Fig. 17.1b).

Although it has been postulated that RT increases the number and activity of CAFs and promotes the accumulation of ECM macromolecules that physically block immune cells trafficking causing an immunosuppressive TME, it was recently demonstrated the benefit of RT strategy in the PACIFIC trial (NCT02125461) (Antonia et al. 2017). In this studio, as we will comment later, RT and immunotherapy are driven synergistically. The lack of recurrences and the improved ability of immune cells to infiltrate and eradicate metastases resulted in extended progression-free survival in patients. Then, RT can modify ECM toward a sketched, reduced tumor stroma and sensitize cancer cells to immune therapies (Menon et al. 2019).

17.5.2 Chemotherapy Induces ECM Remodeling and Promotes the Immune Response

Chemotherapy still represents the core of cancer treatment nowadays. It has been described that this strategy leads to tumor cell death by apoptotic and/or non-apoptotic mechanisms such as autophagy or necrosis (Kerr et al. 1972). For drugs such as cyclophosphamide, gemcitabine, doxorubicin, and cisplatin, the principal mechanism of action described is DNA damage followed by apoptosis (Binotto et al. 2003; Casares et al. 2005). Although apoptosis has been considered as a non-immunogenic cell death, it is now more clear that immunity can be triggered by apoptosis (Nowak et al. 2003). Doxorubicin induces immunogenic apoptosis mediated by the release of the histone HMGB1, which, in turn, activates TLR-4. Also, doxorubicin induces apoptosis by up-regulation of FAS-L in cancer cells (Casares et al. 2005). One possible consequence of chemotherapy on the promotion of immune response has been attributed to alkylating agents. In fact, cyclophosphamide induces the expansion of CD8+ DC, the main subset involved in the cross-presentation of cell-derived antigens (Nowak et al. 2003).

There is experimental evidence that reducing the dose of conventional chemotherapy could act in synergy to generate immunity against many tumors. In this sense, it has been demonstrated that low-dose cyclophosphamide leads to a significant reduction in Tregs population (Malvicini et al. 2009) and paclitaxel reduces the number of MDSCs in a murine model of melanoma (Sevko et al. 2013). Gemcitabine and 5-FU can also selectively deplete MDSCs. It has been described that 5-FU induces MDSC depletion and promotes IFN-γ production by tumor-specific CD8+ T cells in a thymoma model (Vincent et al. 2010). Blidner et al. characterized the effect of indomethacin (IND) on MDSCs and showed that IND inhibited the suppressive

activity exerted by MDSCs on CD8 (+) T cells in lung adenocarcinoma-bearing mice (Blidner et al. 2015). In order to achieve tumor elimination, the immune response should include the activation of cytotoxic T cells that lyse tumor cells. To this end, chemotherapy could work in synergy with ICI therapy.

Focusing on ECM and other factors of the TME, it has been reported that HA production appears to be enhanced in different tumors including ovarian, CRC, breast cancer, and HCC (Freeman and Mielgo 2020). In addition, the presence of high HA levels in TME correlates with poor clinical outcomes. The natural compound 4-methylumbelliferone (4Mu) has been reported to inhibit HA synthesis with antitumor effects in vitro and in vivo (Piccioni et al. 2015). Also, 4Mu therapy reduces HA accumulation and increases the homing of specific antitumor T lymphocytes in CRC-bearing mice (Malvicini et al. 2015).

In addition to HA and GAGs, fibrillar and non-fibrillar ECM components may impact chemotherapy response. The accumulation of ECM proteins is associated with tumor progression and the progressive stiffening of ECM, modifying the sensitivity of cancer cells to chemotherapy. It has been observed that MDA-MB-231 triple-negative breast carcinoma cells exhibited stiffness-dependent resistance response to doxorubicin when were cultured in a 3D alginate-based hydrogel system, and the stiffness-dependent response was lost when cells were removed from 3D to monolayer cultures. These results indicate that ECM conditions are able to define the response to chemotherapy (Joyce et al. 2018). The effects of ECM stiffness on paclitaxel and gemcitabine response were also reported in pancreatic cancer (Amrutkar et al. 2019). The ECM rigidity induces chemoresistance suggesting that TME stiffness would be modulated to overcome resistance and acquire chemotherapy success.

17.5.3 Combined Strategies: Where Are We and Where Are We Going

Chemotherapy in combination with immunotherapy has shown clinical benefit in several tumor types and it is used in clinical practice (Lake and Robinson 2005). Patients with NSCLC were the first ones who benefit from this kind of treatment. The association of pembrolizumab with chemotherapy is used in squamous and non-squamous NSCLC based in the KeyNote 189 and the KeyNote 407 studies, respectively (https://clinicaltrials.gov/). Another interesting combination used in the clinic in this group of patients is chemo plus an anti-PDL1, *atezolizumab*, and an anti-VEGF (*bevacizumab*). All these combinations with platinum-based chemotherapy have shown significant overall survival benefits. The anti-PDL1 has reached its place in the group of patients with metastatic SCLC where *atezolizumab or durvalumab* are being used in combination with platinum plus etoposide. This fact is relevant because SCLC is such a refractory disease.

Another scenario where the chemo-immune combination is used is in esophageal or gastric cancer. The Keynote-590 (https://clinicaltrials.gov/) study showed the combination of cisplatin and 5-fluorouracil plus an anti-PD1 (*pembrolizumab*) improved overall survival over chemotherapy alone. Another anti-PD1 which showed a clinical benefit in combination with chemotherapy in CPS greater than five gastric cancers was *nivolumab*. The OS was 11 vs. 14 months favoring the combination arm. Certainly, there are other studies without so clear results in favor of the combination and these differences could be explained at least in part by the populate selection.

One step forward was done in inoperable stage III NSCLC. The triple combination of chemo-radiotherapy followed by immunotherapy has been approved in this group of patients based on the PACIFIC trial (Antonia et al. 2017). This trial was the first one in demonstrating a significant overall survival and progression-free survival benefit with the addition of immunotherapy after chemo-radiotherapy versus chemo-radiotherapy alone. The overall survival was significantly improved (42.9% vs 33.4%; HR 0.72) as well as progression-free survival (33.1 vs 19%; 0.55) after 5 years of follow-up (America Society of Clinical Oncology meeting, 2021).

There are registered more than 20 phase III clinical trials which combine chemo-, radio-, and immunotherapy. Interestingly, they are proving this triple combination in different ways, like sequential or concurrent; in different pathologies like NSCLC, nasopharyngeal carcinoma, pancreatic cancer, etc.; and in different cancer stages, metastatic or localized ones (https://clinicaltrials.gov/).

17.6 Concluding Comments

A number of immunotherapy strategies have been opened for clinical applications. Nevertheless, the success of these diverse approaches in experimental models is different to the limited outcomes achieved in advanced cancer patients. Immunotherapy could fail or have a slight effect on patient survival for many reasons. The shared scenario for solid tumors often involves patients with advanced diseases that impedes, or at least decreases, the possibility of immunotherapy success. ECM dynamic changes and remodeling are critical for immune cell trafficking, migration, spatial distribution and activation. In the design of an immunotherapeutic approach, the necessity to implement multiple treatments to inhibit TME suppressor mechanisms has to be taken into account. In this context, protocols of combined therapy with focusing in modulate ECM and TME components might act in synergy. Accordingly, conventional chemotherapy and radiotherapy could promote immunity against many tumors. Currently, different forms of immunotherapy including the use of immune checkpoint inhibitors are evaluated in combination along with conventional treatment regimens for overall clinical benefits evidencing improvements in patient survival.

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Chapter 18
Exploiting Hyaluronan-CD44 Network in Tumor Therapy

Theodoros T. Karalis and Spyros S. Skandalis

Abstract Cancer is one of the leading causes of death worldwide. During tumor development and progression, extracellular matrix is being intensively re-organized. One of the most abundant molecules in the extracellular matrix is the polysaccharide hyaluronan. Hyaluronan accumulation and high CD44 expression—the major hyaluronan cellular receptor—correlate with higher malignant states of cancer cells, increased incidence of metastases and poor prognosis of the patients in a wide array of tumor types. Thus, hyaluronan interaction with CD44 emerges as an important target for cancer treatment. In this chapter, recent efforts to exploit hyaluronan/CD44 network for tumor therapy are being discussed. Overall, there is a wide variety of tools available to target this system like anti-CD44 antibodies and peptides, gene therapies against CD44, nanotechnology and modified-hyaluronan. Initial in vivo evidence shows promising results, nominating hyaluronan/CD44 network targeting as a potent candidate to be introduced into clinical settings for tumor therapy.

18.1 Introduction

Cancer is one of the most common-occurring diseases and one of the leading causes of death worldwide. During cancer development, progression, and metastasis, the extracellular matrix is extensively re-organized (Theocharis et al. 2016; Larsen et al. 2006). One of the most abundant molecules in the extracellular matrix is the polysaccharide hyaluronan, which is differentially regulated during tumorigenesis. Physiologically, hyaluronan is found in many tissues such as the skin and the cartilage, where owing to its viscoelastic properties regulates several physiological characteristics of the tissues as well as proper cell function. In cancer, hyaluronan

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metabolism is altered compared to normal tissues and contributes to acquirement of tumor cell malignant properties (Skandalis et al. 2020; Karousou et al. 2017; Heldin et al. 2019). One of the main hyaluronan receptors, CD44, is considered a tumor promoter for most malignancies and plays significant roles in the regulation of cancer cell survival, proliferation, migration, invasion, metastasis, differentiation and drug resistance (Skandalis et al. 2019; Misra et al. 2011; Morath et al. 2016). Given the important roles of hyaluronan and its receptor CD44 in cancer, targeting this system opens a new avenue for the treatment of malignant diseases. Thereby, here we summarize the latest developments in the field of hyaluronan/CD44 network targeting for tumor therapy.

18.1.1 Hyaluronan Synthesis and Catabolism

Hyaluronan is a glycosaminoglycan consisting of repeating disaccharide units of *N*-acetyl-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA), bound through alternate β1–3 and β1–4 linkages (Weigel 2015). Hyaluronan is synthesized in the plasma membrane by specific enzymes termed hyaluronan synthases (HASes). Three hyaluronan synthases have been described in the human genome, *HAS1–3*, which are encoded by distinct genes. *HAS1* is found in 19q13.41 (www.genecards.org, GCID: GC19M054887), *HAS2* in 8q24.13 (www.genecards.org, GCID: GC08M121594) and *HAS3* in 16q22.1 (www.genecards.org, GCID: GC16P069105). Currently there is no information regarding their protein structure, but it is known that they have six transmembrane domains as well as UDP-sugar binding regions (Weigel 2015). Although the three hyaluronan synthases are structurally related they display different spatio-temporal expression and activities. For example, higher expression of HAS1 can be found in adipose tissue and the ovary, HAS2 is highly expressed in the adipose tissue, while HAS3 is expressed in urinary bladder, esophagus, and the lung (proteinatlas.org). Hyaluronan synthases synthesize hyaluronan of different molecular weights. Specifically, HAS2 synthesizes hyaluronan with molecular weight higher than 2×10^6 Da, in contrast to HAS1 and HAS3 which produce hyaluronan ranging from 2×10^5 to 2×10^6 Da (Itano et al. 1999). Furthermore, HAS1 displays the lowest catalytic activity compared to the other HASes, while HAS2 is less active than HAS3. Moreover, HAS1 exhibits higher Km values for both UDP-GlcUA and UDP-GlcNAc compared to HAS2 and HAS3 (Itano and Kimata 2002).

These membrane-embedded glycosyl-transferases utilize as substrates UDP-GlcUA and UDP-GlcNAc which are derived from various metabolic pathways inside the cells (Vigetti et al. 2012; Flores-Diaz et al. 1997). Accordingly, the availability of UDP-sugars is a major factor that regulates hyaluronan biosynthesis (Hascall et al. 2014; Rilla et al. 2013). This in turn suggests that specific microenvironment conditions regulate hyaluronan production, since it has been shown that high glucose upregulates hyaluronan synthesis (Wang et al. 2014). Moreover, the presence of Mg^{2+} is crucial for hyaluronan production (Weigel 2015). The

enzymatic activity of HASes is also regulated by their sub-cellular localization and trafficking from and toward the plasma membrane, where hyaluronan is normally synthesized. HAS1 is mainly localized in Golgi, HAS3 in Golgi and membrane protrusions, while HAS2 is found in the endoplasmic reticulum. The fact that HASes mainly reside inside the cells suggests that there is a reservoir of enzymes ready to translocate in plasma membrane and produce hyaluronan upon stimulation (Torronen et al. 2014). Indicative of this notion is the finding that HAS3 synthesizes a pericellular hyaluronan stroma after its translocation to the plasma membrane (Deen et al. 2014). Furthermore, hyaluronan synthase activity is also regulated by post-translational modifications such as *O*-GlcNAcylation (Vigetti et al. 2012), poly- and mono-ubiquitinylation (Karousou et al. 2010; Mehic et al. 2017) and phosphorylation (Vigetti et al. 2011), while homo- and hetero-dimerization of HAS2 with any of the other synthases leads to concomitant increase in hyaluronan production (Karousou et al. 2010; Bart et al. 2015).

HAS gene regulation is another crucial factor that controls hyaluronan production by the cells in different tissues. Different transcription factors bind to the promoter of each *HAS* gene and control their expression. Specifically, *HAS1* gene contains binding elements for SP1/3, SMAD, and E2F-myc. *HAS2* is regulated by the binding of CREB, NF-κB, RAR, STAT3, YY1, ZEB1, E2F-myc, and SP1, while HAS2-AS1 encoding—a long non-coding RNA that also regulates hyaluronan production by HAS2—is controlled by NF-κB, SP1/3, SMAD and HIF1α. Finally, *HAS3* expression is controlled by binding of ΔN-p63, NF-κB, C/EBP and SP1 in its promoter regions (Heldin et al. 2019).

Furthermore, hyaluronan synthesis is tightly regulated by several growth factors that by inducing intracellular signaling pathways control the expression of several HAS isoforms. The effect of each growth factor on hyaluronan synthesis is cell- and tissue-type-specific. Such growth factors include PDGF-BB, TGF-β, TNFα and IL-1, among others (Heldin et al. 2019).

Hyaluronan amount in the tissues is also modulated by catabolism from specific hyaluronan-degrading enzymes termed hyaluronidases (HYALs). In human, hyaluronan is recycled with high rates. Almost one-third of the total hyaluronan amount can be found in the skin and its half-life ranges from one to one and a half day (Pandey et al. 2008). Hyaluronan in the tissues has initial size about 1000–10,000 kDa and is degraded in the extracellular space in smaller fragments (10–100 kDa) (Fraser et al. 1997). Next, most of these generated fragments are drained through the lymphatic system and degraded in the lymph nodes. The remaining fragments enter the bloodstream and are finally removed by the liver, kidneys, and spleen (Pandey et al. 2008).

In the human genome, several hyaluronidase genes have been found and are encoded by different genes. HYAL-1 and HYAL-2 are widely expressed in several tissues.

HYAL-2 bears a glycosyl-phosphatidyl-inositol (GPI) tail which anchors the protein to the outside of plasma membrane mainly in lipid rafts, together with hyaluronan receptor CD44. HYAL-3 function despite its wide expression has not been fully deciphered (Shuttleworth et al. 2002; Flannery et al. 1998), HYAL-4

degrades chondroitin sulfate (CS) chains, while PHYAL-1 is a pseudogene and is not expressed in human. PH-20/SPAM1 is a hyaluronidase that is expressed in the testis and displays significant hyaluronan degrading activity (Cherr et al. 2001; Baba et al. 2002). Until recently, the widely recognized model for hyaluronan degradation suggested that hyaluronan residing in the extracellular space is degraded initially by HYAL-2 in 20 kDa fragments which then enter the cell by endocytosis through caveolae pathways in endosomes and transported to lysosomes for further degradation to disaccharides by HYAL-1 and exoglycosidases (Montanari et al. 2018). Recently, two new hyaluronidases that need to fit into the scheme of hyaluronan catabolism were discovered, HYBID/CEMIP/KIAA1199 and TMEM2. The transcription and translation of *CEMIP/KIAA1199* gene produces a 153 kDa protein that contains a 30 amino acid N-terminal domain that is required for hyaluronan degradation (Yoshida et al. 2013b). Moreover, it contains seven *N*-glycosylation sites, one G8 domain, two GG, and four PbH1 regions. The GG regions seem to be implicated in the process of hyaluronan degradation, G8 domain in interaction with other proteins and PbH1 in poly-saccharide hydrolysis (Guo et al. 2006; He et al. 2006; Birkenkamp-Demtroder et al. 2011; Yoshida et al. 2013a). Hyaluronan degradation by HYBID is performed by endocytosis in clathrin-coated vesicles with acidic pH, while the resulting hyaluronan fragments are released in the extracellular space (Yoshida and Okada 2019). The *TMEM2* gene product is a transmembrane protein of 154 kDa. The hyaluronidase TMEM2 contains one G8, one GG, and three PbH1 domains in the extracellular region, a transmembrane region, and a cytoplasmic tail (Yamaguchi et al. 2019). In contrast to HYAL-1/-2 which degrade hyaluronan in acidic pH, TMEM2 has optimal pH of enzymatic activity at 6–7. TMEM2 is located in plasma membrane and degrades extracellular hyaluronan in fragments of intermediate size, which then are endocytosed and degraded further in the lysosomes. The enzymatic activity of TMEM2 requires Ca^{2+} as a co-factor (Yamaguchi et al. 2019; Yamamoto et al. 2017). Finally, hyaluronan can be degraded by non-enzymatic ways by the action of reactive oxygen species (ROS) produced by diverse cellular metabolic pathways (Soltes et al. 2006; Agren et al. 1997).

18.1.2 CD44

Hyaluronan synthesized in the plasma membranes is subsequently extruded to the extracellular space where it can interact with several extracellular proteins, such as the proteoglycan family hyalectans, or plasma membrane receptors, and thus regulate several cell functional properties. The hyaluronan receptors discovered so far include CD44 (Cluster of Differentiation 44), RHAMM (Receptor for Hyaluronan-Mediated Motility), HARE/STAB2 (Hyaluronic Acid Receptor for Endocytosis/Stabilin-2), Laylin, Stabilin-1 and LYVE-1 (Lymphatic Vessel Endothelial Hyaluronic Acid Receptor 1).

The major and best characterized receptor for hyaluronan is CD44, which is expressed in various cell types and tissues. *CD44* gene is located in 11p13

(genecards.org) and its transcript mRNA is subjected to alternative splicing which after translation leads to encoding of several different isoforms of the receptor. Human *CD44* gene contains 19 exons, 10 standard (S1–10) and 9 variants (V2–10). The S1–10 exons are retained in all CD44 isoforms. CD44s (CD44 standard) isoform does not contain any of the V2–10 exons, while alternative splicing of V2–10 gives rise to CD44v (CD44 variants). From the mRNA translation the final product is a transmembrane protein with an extracellular region containing a LINK domain—which is responsible for hyaluronan/CD44 interactions—and a stalk-like domain—in which the exons V2–V10 are introduced—a transmembrane region, and a small cytoplasmic tail. The extracellular domain is modified by *O*- and *N*-glycosylations, while specific CD44 isoforms, such as CD44v3, bear covalently bound glycosaminoglycan chains (chondroitin sulfate or heparan sulfate chains), contributing further diversity to the resulting proteins. The cytoplasmic tail of CD44 despite not containing intrinsic kinase activity regulates several signaling pathways through interactions with cytoplasmic proteins such as Src, ERM (Ezrin, Radixin, Moesin) and IQGAP1 (Zoller 2011; Skandalis et al. 2010). Moreover, CD44 intracellular domain can be cleaved by γ-secretase and translocated to the nucleus where it regulates the expression of several genes (e.g., MMP9) (Miletti-Gonzalez et al. 2012). Apart from cellular signaling, CD44 can also participate in the endocytosis of hyaluronan, leading to its degradation (Thankamony and Knudson 2006; Skandalis et al. 2020).

18.2 Roles of Hyaluronan-CD44 Network in Tumors

Apart from the diverse roles of hyaluronan and its receptor CD44 in physiological processes like embryogenesis and cartilage function they play significant roles in tumor development and progression. In tumors, hyaluronan creates a highly hydrated extracellular matrix with specific physicochemical properties which allows cancer cells to proliferate and migrate. Moreover, hyaluronan synthesized by tumor stromal cells or cancer cells themselves, engages CD44 on the surface of cancer cells to regulate biological processes like growth/survival, epithelial-to-mesenchymal transition (EMT), differentiation, invasion, metastasis, drug resistance and cancer stem cell properties.

18.2.1 Growth/Survival

For tumors to successfully form, cancer cells need to deal with several stressful events, such as anchorage-independent growth, hypoxia and limited nutrient availability. Therefore, it is critical for cancer cells to take advantage of physiological molecular mechanisms and pathways allowing them to cope with such stressful events. One of the major cellular receptors correlated with survival and

anti-apoptotic signaling in cancer cells is the hyaluronan receptor CD44. CD44 regulates the expression and activation of proteins involved in resistance to apoptosis and cell growth like Fas, caspase 3/9, Bcl-xl/Bak, Akt, pRb and Bcl-2 (Lakshman et al. 2004; Yasuda et al. 2001; Park et al. 2012b). For example, overexpression of CD44s, CD44v3–10, and CD44v8–10 in human colon cancer cells successfully attenuated etoposide-induced cell death (Lakshman et al. 2004). On the other hand, inhibition of CD44 expression in colon carcinoma cells reduced the expression of Bcl-2, Bcl-xL, while simultaneously increased the expression of apoptosis proteins Bax and caspase-3/8/9 (Park et al. 2012b). Furthermore, CD44 controls downstream activation of Akt, a major survival pathway, and cell cycle-regulating proteins p21 and pRb in many types of cancer cells, like breast, colon and lung cancer cells (Lakshman et al. 2004). In lung cancer cells, interaction of hyaluronan with CD44 reduced Fas expression and subsequent Fas-mediated apoptosis (Yasuda et al. 2001). In chronic lymphocytic leukemia (CLL) patients CD44 was found to promote cancer cell survival. The crucial importance of CD44 in CLL was further certified by the fact that CD44 knock-down reduced survival even in Akt-overexpressing cells. In that model, CD44 regulated the expression of MCL1 anti-apoptotic protein through Akt and Erk pathways (Fedorchenko et al. 2013). Hyaluronan engaged to CD44 also induced phosphorylation of FAK, which associates with PI3K to protect against apoptosis (Fujita et al. 2002). Furthermore, hyaluronan-CD44 interaction activated ErbB2 signaling through Hsp90, cdc37, p110 and p85 proteins (Chanmee et al. 2015).

18.2.2 Epithelial-to-Mesenchymal Transition (EMT) and Differentiation

Epithelial-to-mesenchymal transition is a dynamic process that cancer cells utilize in order to metastasize. During this process, hyaluronan synthase expression is induced and hyaluronan is synthesized to large amounts. Moreover, CD44 has been found to be overexpressed in mesenchymal cancer cells and its high expression correlates with a more undifferentiated phenotype (Misra et al. 2011; Heldin et al. 2014). In breast cancer, EMT correlates with poor prognosis and intriguingly, breast cancer cells with mesenchymal and more malignant phenotype express higher amounts of CD44, which also displays high hyaluronan-binding capacity (Bernert et al. 2011; Heldin et al. 1996). In breast epithelial cultures, TGF-β-induced EMT depends on the expression of HAS2. Specifically, TGF-β induces Smad and p38 MAPK pathways to upregulate HAS2 expression (Porsch et al. 2013). Moreover, HAS2 overexpression has been shown to promote the malignant phenotype via suppression of E-cadherin and translocation of β-catenin to the nucleus, signaling events that take place during EMT (Zoltan-Jones et al. 2003; Koyama et al. 2007). Interestingly, switching between CD44v isoforms to CD44s through alternative splicing promoted EMT by suppression of E-cadherin through PI3K/Akt pathways. Inhibition of splicing activity that produced CD44v occurred through downregulation of epithelial

splicing regulatory protein 1 and 2 (ESRP1 and 2) by transcription factors Snail1, Zeb1, and Zeb2 (Reinke et al. 2012). Expression of CD44 and its interaction with hyaluronan also regulate differentiation of aggressive cancer cells like acute myeloid leukemia cells, thus downregulating their aggressive properties (Solis et al. 2012).

18.2.3 Invasion/Metastasis

Accumulation of hyaluronan as well as high CD44 expression in the cancerous tissues has been widely correlated with advanced incidence of invasion and metastasis of various types of cancer cells. CD44 interacts with several growth factor receptors like ErbB2 and PDGFR and its interaction with hyaluronan regulates their signaling activity (Bourguignon et al. 1997; Ghatak et al. 2005; Li et al. 2006). Binding of hyaluronan induces CD44 clustering, which activates downstream signaling pathways in a cell- and tissue-dependent manner. This clustering was critical for MMP9 activation and subsequent activation of TGF-β, which in turn induced cancer cell invasion and metastasis (Yu and Stamenkovic 1999). Moreover, CD44 interacted with MT1-MMP—a major metalloproteinase responsible for extracellular matrix degradation during invasion and metastasis—to enhance its activity. In turn, MT1-MMP enhanced the shedding of CD44 variants and promoted cancer cell invasiveness (Kajita et al. 2001; Stamenkovic and Yu 2009; Mori et al. 2002). Hyaluronan fragments also have a functional role during invasion and metastasis. Specifically, hyaluronan dodecasaccharides by engaging CD44 induced secretion of CXCL1, to enhance endothelial cell sprouting, which could be critical for tumor angiogenesis, a key process in metastasis (Takahashi et al. 2005). Hyaluronan is also synthesized by stromal cells. This stromal cell-derived hyaluronan engaged CD44v6 in the surface of colon tumor cells to sustain PI3K signaling in a positive CD44v6/PI3K loop and promote invasion (Misra et al. 2011).

18.2.4 Drug Resistance

One of the main properties that cancer cells acquire during tumor development is resistance to several drugs. Drug resistance in the tumors is thought to be acquired through different mechanisms. First, a subpopulation of cells with pre-existing potential for drug resistance could be present inside a tumor. Second, drugs utilized for cancer therapy can induce resistance by cancer cells. In both cases, cancer cells upregulate the expression of proteins, like multi-drug resistance proteins (MDRs) that allow them to evade drug-induced apoptosis. Intriguingly, there is extensive evidence correlating CD44 expression with resistance to radiotherapy or chemotherapy of various types of cancer cells (Yaghobi et al. 2021). CD44 physically interacts with P-glycoprotein to enhance drug resistance in cancer cells (Miletti-Gonzalez et al. 2005). In malignant cells, decrease in CD44 expression ameliorated drug

resistance (Xu et al. 2015). Specifically, in hepatocellular carcinoma, inhibition of CD44 sensitized cancer cells to sorafenib (Fernando et al. 2015). Paclitaxel-resistant ovarian cells also showed increased expression of CD44 (Gao et al. 2015). It is important to note that interaction of high molecular weight hyaluronan with CD44 increased MDR expression and subsequently led to drug resistance, while low molecular weight hyaluronan-CD44 interaction led to MDR internalization, signifying that not only the presence of hyaluronan but also its size is critical for this process (Zoller 2011). Moreover, hyaluronan through CD44 binding activated PI3K signaling, which in turn induced MDR protein expression (Misra et al. 2005). In the context of CD44v, CD44v3 targeting in head and neck squamous cell carcinoma cells reduced resistance to cisplatin (Wang et al. 2007). Hyaluronan-CD44v3 interaction upregulates miRNA-302, enhanced Oct4-Sox2-NANOG signaling and increased expression of MDR1, leading to development of chemo-resistance (Bourguignon et al. 2012). CD44v3-hyaluronan interaction also regulated expression and activity of P300 which can in turn acetylate β-catenin and NF-κB-p65, resulting in upregulation of MDR1 expression (Zoller 2015). Interestingly, in prostate cancer, knocking down the expression of CD44v6 enhanced chemotherapy sensitivity (Ni et al. 2014).

18.2.5 Tumor Stem Cell Properties

In tumors, sub-populations of cancer cells, termed cancer stem cells (CSCs), have been shown to be responsible for tumor recurrence after chemotherapy or radiotherapy. Hyaluronan, like in normal stem cell niches, creates an ideal micro-environment for CSCs survival, self-renewal, and maintenance. Importantly, hyaluronan receptor CD44 is widely recognized as a stem cell marker (Skandalis et al. 2019). $CD44^{High}CD24^{Low}$ populations isolated from tumors of the breast showed stem cell properties, like self-renewal and tumor-initiating capacity (Shao et al. 2016; Li et al. 2017b; Wei et al. 2012). Mechanistically, ΔNp63 induced the expression of HAS3, HYAL-1, and CD44 to create a hyaluronan-rich environment that favored stemness of breast cancer cells (Gatti et al. 2018). The importance of hyaluronan and CD44 was further solidified by experiments in hyaluronan-based multilayer nanofilms, where pancreatic cells grown in such conditions upregulated CD44v6 and form colonies (Lee et al. 2018). In pancreatic tumor cells, the tumor suppressor KFL4 bound to CD44 promoter to block its expression and ameliorated cancer stem cell properties and metastasis (Yan et al. 2016). On the other hand, targeting hyaluronan with 4-methyl-umbelliferone—a widely utilized hyaluronan synthesis inhibitor—promoted phagocytosis of hepatocellular CSCs (Rodriguez et al. 2018). CD44 also activated Wnt/β-catenin pathway to upregulate FoxM1 and Twist, leading to enhanced stemness of lung adenocarcinoma cells (Su et al. 2016). Circulating oral squamous carcinoma cells that display CD44 expression were able to form spheres and displayed chemoresistance and self-renewal (Patel et al. 2016). For a more comprehensive analysis of hyaluronan/CD44 roles in cancer stem cell properties, refer to our previous review (Skandalis et al. 2019).

18.3 Hyaluronan/CD44 Network Targeting

Given the important roles of hyaluronan/CD44 network in tumor development, progression, and metastasis, this system emerges as a potent pharmacological target. Therefore, several efforts have been concentrated toward this purpose, such as incorporation of hyaluronan on nanoparticles for specific delivery of drugs to tumor cells, antibodies or peptides blocking CD44 actions and interactions, chemically modified hyaluronan, and utilizing gene therapies (CRISPR/Cas9 or sh/siRNAs) against CD44 (Fig. 18.1). Below, we describe recent advances in pharmacological targeting of this system for cancer treatment (summarized in Table 18.1).

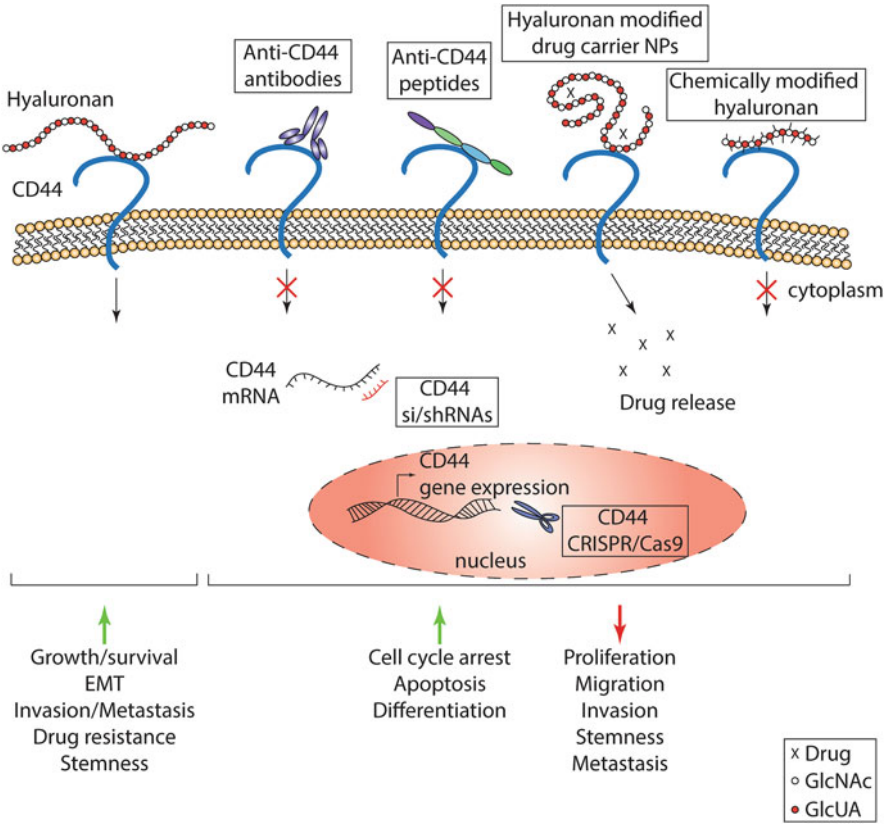

Fig. 18.1 Hyaluronan/CD44 network targeting approaches. Incorporation of hyaluronan on nanoparticles for specific delivery of drugs to tumor cells, antibodies or peptides blocking CD44 actions and interactions, chemically modified hyaluronan and gene therapies targeting CD44 mRNA (CRISPR/Cas9 or sh/siRNAs against CD44)

Table 18.1 Overview of hyaluronan/CD44 targeting tools (for further details, see text)

Approach	Tool	Tumor	Function	References
Nanomedicine	HA-PEI-PLGA-docetaxel NPs	Lung	↓ Proliferation	Maiolino et al. (2015)
	HA-PLGA-docetaxel NPs	Lung	↓ Tumor growth	Wu et al. (2017)
	HA-Dextran-modified quercetin-Cu(II) NPs with PARP inhibitor	Breast	↑ Animal survival	Cheng et al. (2021)
	HA-Resverastrol/chalcone NPs	Breast	↑ Drug cytoplasmic release	Shah et al. (2017)
	HA-Doxorubicin-super paramagnetic iron oxide NPs	Breast	↑ Drug uptake and release, apoptosis	Vyas et al. (2015)
	HA-Berberine derivative/doxorubicin NPs	Breast	↑ Mitochondrial apoptotic pathways ↓ Proliferation, migration, lung metastasis	Lin et al. (2021)
	HA-Chitosan-docetaxel NPs	Breast	↓ Cell viability	Shabani Ravari et al. (2016)
	HA-Curcumin/celecoxib NPs	Breast	↑ Apoptosis, animal survival ↓ Tumor growth, cell viability, lung metastasis	Liu et al. (2020)
	HA-Paclitaxel/curcumin PAAVB NPs	Breast	↑ Adaptive anti-tumor immunogenicity ↓ Immunosuppression, lung metastasis	Wang et al. (2021)
	HA-(PANi)-imiquimod NPs	Breast	↑ Immune responses against tumor	Yasothamani et al. (2021)
	HA-Salinomycin/curcumin NPs	Breast (CSCs)	↑ G1 cell cycle arrest ↓ EMT	Zhao et al. (2020)
	HA-NPs with saporin	Breast	↑ Apoptosis	Ding et al. (2018)
	HA-Hyaluronidase/Ce6 NPs	Breast	↑ Uptake, efficacy	Gong et al. (2016)
	HA-Gold-nanoclustered verteporfin NPs	Breast	↑ Blood stream stability, drug delivery ↓ Tumor growth	Han et al. (2016)
	Multifunctionalized iron oxide magnetic nanoparticles (MNPs) with anti-CD44 antibody and gemcitabine derivatives	Breast and pancreatic	↑ Cancer cell killing	Aires et al. (2016)
	HA-Cisplatin green tea catechin micelles	Ovarian	↑ Drug efficacy ↓ Off-target effects	Bae et al. (2017)

	Chitosan polylactic acid- coated with IM7 anti-CD44 antibody	Ovarian	↓ Toxicity	Yang et al. (2017)
	HA-Serum albumin- conjugated NPs	Ovarian	↑ Drug uptake, cytotoxicity against cancer cells	Edelman et al. (2017)
	HA-Paclitaxel NPs	Colon	↑ Animal survival	Zhu et al. (2018)
	HA-PTC209 NPs	Colon (CSCs)	↓ Tumor growth	Xu et al. (2019)
	HA-Ce6 phosphosensitizer NPs	Colon	↓ Tumor growth	Gao et al. (2017)
	HA-poly(hexamethylene biguanide) and chitosan NPS with KRAS si/shRNA	Colorectal	↑ Uptake and KRAS silencing	Tirella et al. (2019)
	HA-Doxorubicin lipoic-acid encapsulated NPs	Myeloma and acute myeloid leukemia	↓ Tumor growth	Zhong et al. (2017)
	A6 anti-CD44 peptide polymersomal epirubicin	Multiple myeloma	↑ Uptake, anti-cancer efficacy	Gu et al. (2019)
	HA-Cabazitaxel/Silibinin liposomes	Prostate (CSCs)	↑ Apoptosis ↓ Migration	Mahira et al. (2019)
	CDDP NPs	Prostate (CSCs)	↓ Stem cell properties	Jafari Malek et al. (2014)
	HA-Diethyldithiocarbamate- copper NPs	Pancreatic (CSCs)	↑ ROS generation ↓ Sphere formation	Marengo et al. (2019)
	HA-chitosan NPs with Bcl- 2 siRNA	Bladder	↓ Bcl-2 expression	Liang et al. (2021)
	HA-HPLR- with EGFR targeted peptides	Liver	↓ Tumor growth	Liang et al. (2019)
	HA NPs with Gli1 siRNA	Gastric (CSCs)	↓ Migration, invasion, tumor spheroid, and colony formation	Yao et al. (2020)
	HA-docetaxel NPs with AS1411 aptamer	Glioblastoma	↑ Blood-brain barrier penetration, drug uptake ↓ Spheroid formation, tumor growth	Wang et al. (2019)
	HA-Doxorubicin liposome NPs	Glioblastoma	↑ Anti-neoplastic function, doxorubicin efficacy	Hayward et al. (2016)
	HA-Zinc protoporphyrin nanoprobes	Sarcoma	↑ Anti-cancer effect ↓ Side effects	Gao et al. (2021)

(continued)

Table 18.1 (continued)

Approach	Tool	Tumor	Function	References
Antibodies	RG7356	Chronic lymphocytic leukemia	↑ Apoptosis	D'arena et al. (2014) and Zhang et al. (2013)
		Breast	↑ Secretion of immune cell chemo-attractants, antibody-dependent cellular phagocytosis ↓ Tumor growth	Weigand et al. (2012) and Maisel et al. (2016)
	A3D8	Acute myeloid leukemia	↑ G0/G1 cell cycle arrest	Zada et al. (2003), Gadhoum et al. (2004a, b), and Qian et al. (2012)
		Chronic lymphocytic leukemia	↑ Caspase activation ↓ Cell viability	Fedorchenko et al. (2013)
		Erythroleukem ia	↑ Caspase-independent apoptosis ↓ Cell growth	Artus et al. (2006)
		Ovarian (CSCs)	↑ S phase cell cycle arrest, apoptosis	Du et al. (2013)
	F77	Prostate	↑ Apoptosis	Chen et al. (2018b)
	Hermes-1	Colorectal carcinoma	↓ Binding of hyaluronan, laminin and collagen	Ishii et al. (1993)
	IM7	Colorectal carcinoma	↓ Liver metastasis	Ogoshi et al. (1998)
		Glioblastoma	↑ Apoptosis ↓ Hyaluronan biosynthesis	Wiranowska et al. (2010)
		Ovarian	↓ Proliferation, tumor development, and progression	Yang et al. (2017)
		Bladder	↓ NF-κB activation	Fitzgerald et al. (2000)
		Melanoma	↓ Adhesion of melanoma cells on endo-thelial cells	Ota et al. (1995)
	IM7 and S5	Natural killer cells	↑ Natural killer cell activity	Tan et al. (1993)
	IM7 conjugated to saporin	Prostate	↑ Apoptosis	Bostad et al. (2014)
	J173 and F10442	Natural killer cells	↑ CD16-mediated lysis, intracellular Ca^{2+}	Galandrini et al. (1994)

	J173	Burkitt's lymphoma and chronic myelogenous leukemia	↑ Killing activity of peripheral mononuclear cells against cancer cells	Ishizuka et al. (2008)
	HI44α	Acute myeloid leukemia	↑ Differentiation, apoptosis	Song et al. (2004)
	KMP1	Bladder	↓ Cell proliferation, migration and adhesion, tumor growth	Chen et al. (2018c)
	aCD44O^{2}LNCs with paclitaxel	Pancreatic (CSCs)	↑ Anti-tumor efficacy	Navarro-Marchal et al. (2021)
	5-mG2a-f	Oral squamous cell carcinoma	↓ Tumor development	Takei et al. (2020)
	P3D2	Breast	↓ Tumorigenesis	Lusche et al. (2021)
	Anti-CD44v9 mAb 44-IV	Colon	↓ Cancer cell capillary adhesion, liver metastasis	Seki et al. (1997)
Peptides	RP-1	Gastric	Detection of CD44$^+$ cancer cells, prognosis prediction	Zhang et al. (2015) and Li et al. (2017a)
	CV-1	Gastric	Detection of CD44v3–10$^+$ cancer cells	Zhang et al. (2016)
	PFT	Prostate	Detection of CD44v6$^+$ cancer cells	Peng et al. (2017)
	PDPP	Breast	Detection of CD44$^+$ CSCs	Cho et al. (2015)
	A6	Chronic lymphocytic leukemia	↑ Toxicity	Finlayson (2015)
		Ovarian and breast	↓ Migration	Piotrowicz et al. (2011)
		Melanoma	↓ Lung metastasis	Piotrowicz et al. (2011)
	A6 with reduction-sensitive polymersomal vincristine sulfate	Acute myeloid leukemia	↑ Animal survival ↓ Leukemia burden in the circulation, bone marrow, liver, and spleen	Gu et al. (2021)
	v6	Pancreatic	↑ Animal survival ↓ Tumor growth	Matzke-Ogi et al. (2016)
	NLN and NEW	Breast and pancreatic	↑ CD44v6 internalization ↓ c-Met/Erk activation, migration, invasion, metastasis	Khan et al. (2021)

(continued)

Table 18.1 (continued)

Approach	Tool	Tumor	Function	References
	NLN and NEW with KLA pro-apoptotic peptides	Breast and pancreatic	↑ Tumor cell killing ↓ Tumor growth, metastasis, side effects	Khan et al. (2021)
	FKBPL and AD-01	Breast and prostate	↑ Anti-angiogenic effects ↓ Tumor growth	Valentine et al. (2011)
		Breast	↓ Migration	Yakkundi et al. (2013)
	AD-01	Breast (CSCs)	↑ Differentiation ↓ Mammosphere formation, tumor initiation, chemotherapy, and radiotherapy resistance when combined with DAPT	McClements et al. (2013)
	Peptides derived from collagen type IV with liposomes and doxorubicin	Melanoma	↓ Tumor size	Ndinguri et al. (2012)
	P3 and P9	Chronic lymphocytic leukemia	↓ Adhesion to pro-MMP9, chemotaxis, transendothelial migration	Ugarte-Berzal et al. (2014)
	PCK3145	Fibrosarcoma	↓ Adhesion to hyaluronan	Annabi et al. (2005)
	A5G27	Melanoma	↓ Metastasis	Hibino et al. (2005)
	A5G27 with siRNA particles	Lung and ovarian	↓ Tumor growth	Golan et al. (2016)
	CD44BP	Breast	↓ Tumor sphere formation	Yang et al. (2013)
	CD44 cytoplasmic tail peptides bearing phosphor- $_{Ser}$325	Melanoma	↓ Migration	Peck and Isacke (1998)
	CD44-derived peptides as immunogens	Prostate (CSCs)	↑ Sensitize dendritic cells, anti-tumor activities	Wang et al. (2020)
Chemically modified hyaluronan	Modification with Au-Ag alloy in NPs	Breast	↑ Sensitivity to radiotherapy	Chong et al. (2020)
	Modification with a hydrazide group and bisphosphonate (BP)	Colon	↑ Targeting	Varghese et al. (2009)
	Modification with sulfate groups	Pancreatic	↑ Targeting of CD44 and P-selectin	Bhattacharya et al. (2020)

	Modification with poly(lactic-*co*-glycolic acid) and loaded to transferrin- targeted nanoformulated AUY922 NPs	Brain	↑ Uptake, apoptosis, side effects ↓ Tumor growth	Debele et al. (2021)
	Modification with β-cyclodextrin and drug conjugates	Lung and prostate	↑ Toxicity against cancer cells ↓ Toxicity against normal cells	Bai et al. (2020)
Gene therapies	CD44 siRNA	Lung	↓ Stem cell gene expression	Nurwidya et al. (2017)
	CD44 siRNA in combination with doxorubicin	Breast	↑ Apoptosis ↓ Tumor metastasis, proliferation, invasion, migration	Vahidian et al. (2020)
	CD44 siRNA in liposomes with Apt1 CD44-targeted aptamer	Breast	↓ CD44 expression	Alshaer et al. (2018)
	CD44 shRNA	Breast	↓ Proliferation, colony formation, invasion	Zhou et al. (2018)
	PLGANPs with FAK and CD44 siRNAs	Ovarian	↑ Apoptosis ↓ Tumor size, angiogenesis, proliferation	Zou et al. (2013)
	CD44 shRNA adenovirus	Colon	↑ Apoptosis ↓ Proliferation, migration, invasion	Lee et al. (2017)
	CD44v6 shRNA	Colon	↓ Adenoma growth	Misra et al. (2009)
	CD44 CRISPR/Cas9	Osteosarcoma	↓ Proliferation, spheroid formation, migration, invasion	Liu et al. (2018)
		Osteosarcoma	↑ Drug sensitivity of drug- resistant cells	Xiao et al. (2018)
		Liver (CSCs)	↑ Tumor differentiation ↓ Tumor malignancy	Han et al. (2015)
	CD44v6 CRISPR/Cas9	Gastric	↑ Cisplatin sensitization ↓ Self-renewal	Lobo et al. (2020)
	Cosmc CRISPR/Cas9	Breast	↓ CD44 O-glycosylation and expression, proliferation	Du et al. (2020a)

HA hyaluronan, NPs nanoparticles, CSCs cancer stem cells

18.3.1 Nanomedicine

Nanomedicine therapies utilize targeted drug delivery approaches to specifically target tumor cells and avoid adverse effects in normal tissues. Accordingly, there are two types of targeted drug delivery. First, passive targeting, which takes advantage of enhanced permeability and retention effect (EPR) in tumors. The EPR effect allows for accumulation of drugs and nanoparticles in tumors that have distinct architectural features from normal tissues, such as hyper-vasculogenesis, impaired lymphatic drainage, and different abnormal interstitial pressures (Fang et al. 2011). Second, active targeting, follows passive targeting to selectively deliver specific drugs to cancer cells overexpressing the desired receptor, taking advantage of receptor-substrate interactions (Danhier et al. 2010). Due to the important roles of hyaluronan/CD44 interactions and the correlation between CD44 expression and higher states of malignancy, hyaluronan is widely utilized in nanomedicine to specifically target CD44-expressing cancer cells and cancer stem cells. In blood circulation drug bioavailability and activity is altered by the body's defense, through chemical modification or binding to serum factors. In the last years, a "3S" transition concept has emerged, which incorporates stability transition, surface transition and size transition to overcome barriers in the delivery process. Hyaluronan therefore is an excellent candidate to be incorporated to nanoparticles and drug carriers, since it fulfills all three criteria due to its biocompatibility, biodegradability, and specific targeting of CD44-expressing cancer cells (Zhong et al. 2020).

One of the main purposes of hyaluronan-coated nanoparticles is to specifically deliver encapsulated drugs in cancer cells to enhance anti-tumor efficacy and avoid adverse effects. Hyaluronan can decorate polyetheleneimine (PEI) biodegradable nanoparticles of poly(lactic-*co*-glycolic) acid (PLGA) to deliver docetaxel specifically to lung cancer cells and inhibit their proliferation (Maiolino et al. 2015). Tumor growth inhibition of lung cancer cells in vivo was also observed by using PLGA hyaluronan-coated docetaxel nanoparticles without PEI, verifying the anti-cancer efficacy of this strategy (Wu et al. 2017). In another study, docetaxel loaded in chitosan-coated hyaluronan nanoparticles was more effective than free docetaxel against $CD44^+$ breast cancer cells (Shabani Ravari et al. 2016). Hyaluronan nanoparticles composed of branched cell-penetrating peptide B-mR9 could also successfully deliver methotrexate to $CD44^+$ cells and exerted antitumor activity (Yoo et al. 2020). Curcumin and celecoxib loaded in hyaluronan-coated nanoparticles displayed in vitro toxicity and inhibited tumor growth, enhanced survival of mice, induced apoptosis, and abrogated the formation of lung metastasis of breast cancer cells (Liu et al. 2020). Tumor cells display higher levels of glutathione. Taking advantage of this, hyaluronan-coated redox-sensitive micelles were developed to aim CD44-expressing tumor cells and release the drugs inside the target cells (Du et al. 2020b). Furthermore, dextran-modified quercetin-Cu(II)/hyaluronan nanoparticles with a natural PARP inhibitor could induce synthetic lethality in triple-negative breast cancer cells and extended animal survival, without displaying any adverse effects on normal organs (Cheng et al. 2021). Similarly, in

triple-negative breast cancer cells conjugation of resveratrol and a chalcone to hyaluronan enhanced their uptake (Shah et al. 2017). Doxorubicin was loaded in hyaluronan-super paramagnetic iron oxide nanoparticles to achieve better uptake and cytoplasmic release of the drug and subsequently enhanced apoptosis in triple-negative breast cancer cells (Vyas et al. 2015). In addition, hyaluronan could facilitate lipoplexes delivery in breast cancer cells that express CD44 (Surace et al. 2009). Recently, targeting mitochondrial pathways in cancer cells has emerged as a promising strategy for tumor therapy. Indeed, hyaluronan nanomedicine utilizing a berberine derivative and doxorubicin was able to target mitochondria and subsequently inhibited proliferation, migration, and enhanced apoptosis of triple-negative breast cancer cells. Importantly, lung metastasis in vivo was abrogated after this treatment. These effects were mediated by suppression of MMP-2/-9 activities and induction of mitochondrial apoptotic pathways (Lin et al. 2021). Cisplatin can be also loaded to hyaluronan-green tea catechin micellar nanocomplexes, to enhance its efficacy against ovarian cancer cells, without causing side effects in vivo (Bae et al. 2017). In glioblastoma cells, hyaluronan-conjugated liposome nanoparticles effectively delivered doxorubicin inside the cells and enhanced its anti-neoplastic function (Hayward et al. 2016). Paclitaxel is another drug that can be also delivered with hyaluronan-nanoparticle complexes to treat CD44-positive colon cancer cells in orthotopic mouse models (Zhu et al. 2018). Paclitaxel and curcumin encapsulated in poly (acrylamide-*co*-acrylonitrile-*co*-vinylimidazole-*co*-bis(2-methacryloyl) oxyethyl disulfide) (PAAVB) polymer-based intelligent platform coated with hyaluronan, were able to promote adaptive anti-tumor immunogenicity and inhibit immunosuppression of CD44-overexpressing breast tumor cells while simultaneously abrogating lung metastasis (Wang et al. 2021). Hyaluronan can also decorate serum albumin conjugate-based nanoparticles to enhance drug delivery and their cytotoxic effects on cancer cells expressing CD44 (Edelman et al. 2017). In addition, promising results have been obtained by using hyaluronan-modified nanoparticles for treatment of hematological tumors, where CD44 plays significant roles. Specifically, doxorubicin encapsulated in lipoic acid-crosslinked hyaluronic acid nanoparticles was used to inhibit tumor growth of multiple myeloma and acute myeloid leukemic cells in vivo (Zhong et al. 2017).

The size of hyaluronan that may be used to coat nanoparticles is apparently important. Specifically, hyaluronan nanoparticles coated with low molecular weight hyaluronan displayed low CD44-binding activity, while high binding affinity was displayed by high molecular weight hyaluronan-coated nanoparticles, suggesting that high molecular weight hyaluronan is more suitable as a coating substance (Mizrahy et al. 2011). Furthermore, high molecular weight hyaluronan-coated nanoparticles displayed enhanced circulation time and better tumor targeting specificity than low molecular weight hyaluronan-coated nanoparticles (Mizrahy et al. 2014).

As already mentioned, CD44 serves as a major cancer stem cell marker. Therefore, hyaluronan can be used to guide nanoparticle-drug conjugates to cancer stem cells. To target prostate cancer stem cells, hyaluronan was used to decorate cabazitaxel and silibinin co-encapsulated cationic liposomes to induce apoptosis

and decrease cell migration (Mahira et al. 2019). Cis-dichlorodiamminoplatium (II) (CDDP) glyconanoparticles were also coated with hyaluronan to deliver the drug in vivo and suppress stem cell properties of prostate cancer cells (Jafari Malek et al. 2014). For breast cancer treatment, co-delivery of salinomycin and curcumin in hyaluronan-coated nanoparticles was achieved in CD44-expressing breast cancer stem cells, inducing cell cycle arrest in G1 and inhibiting EMT (Zhao et al. 2020). To target pancreatic cancer stem cells, hyaluronan was utilized to coat diethyldithiocarbamate-copper complex nanoparticles. These nanoparticles were able to impair sphere formation of pancreatic cancer stem cells, probably by inducing the generation of ROS (Marengo et al. 2019). Nanoparticles can be delivered also orally. Specifically, hyaluronan-decorated nanoparticles containing PTC209, a BMI-1 inhibitor, were able to target colon cancer stem cells and abrogate tumor growth in vivo (Xu et al. 2019).

Hyaluronan-coated nanoparticles can encapsulate phosphosensitizers, like Ce6, to deliver them in human colon cancer cells in vivo. After photodynamic therapy, tumor growth was attenuated, while no side effects were observed (Gao et al. 2017). Furthermore, co-administration of hyaluronidase with such nanoparticles induced their uptake from tumor cells and enhanced their anti-cancer efficacy, probably by increasing the enhanced permeability and retention (EPR) effect inside the tumor (Gong et al. 2016). For triple-negative breast cancer treatment, photothermally targeted hyaluronan-polyaniline (PANi)-imiquimod (R837, a TLR7 agonist) nanoparticles were used to induce immune responses against the tumor (Yasothamani et al. 2021). Interestingly, hyaluronan-decorated nanoparticles could be effectively used to encapsulate and deliver hyaluronidase enzymes specifically to the tumors together with doxorubicin to enhance its efficacy (Chen et al. 2018a). Gold-nanoclustered hyaluronan nano-assemblies were also used as a platform for photodynamic or photothermal cancer therapies, by delivering the photodynamic therapy agent verteporfin in cancer cells. These nanoparticles displayed excellent stability in the blood and were able to completely inhibit tumorigenesis while displaying 100% survival rate (Han et al. 2016). Hyaluronan-conjugated zinc protoporphyrin nanoprobes were also used for photodynamic therapy against colon cancer and fibrosarcoma cells (Gao et al. 2021).

Among others, hyaluronan-coated nanoparticles can carry siRNAs or shRNAs against specific proteins expressed in cancer cells. KRAS is a known oncogene with no available small molecule inhibitors. To target KRAS hyaluronan-decorated nanocarriers [poly(hexamethylene biguanide) and chitosan] bearing KRAS-si/shRNA have been designed to specifically silence KRAS expression in CD44-expressing cancer cells (Tirella et al. 2019). Moreover, chitosan nanoparticles were conjugated with hyaluronan to target bladder cancer cells that expressed high levels of CD44, to deliver siRNA against the Bcl-2 oncogene. These particles were able to target bladder cancer cells in vivo and successfully interfere with the Bcl-2 expression (Liang et al. 2021). A new anti-cancer siRNA delivery system named by the authors HPLR was manufactured with a siRNA-peptide core surrounded by lipid bilayer, thin hyaluronan coating, and EGFR-targeted peptides to target EGFR and CD44 overexpressing cells. This system was able to successfully deliver the siRNAs

in subcutaneous liver tumors and inhibit their growth without displaying any significant toxicity (Liang et al. 2019). Additionally, hyaluronan-conjugated nanoparticles were utilized to deliver Gli1 siRNA in gastric cancer stem cells and subsequently reduced migration, invasion as well as tumor spheroid and colony formation (Yao et al. 2020).

Notably, nanoparticles can also be decorated with CD44-targeting peptides. Indeed, A6 anti-CD44 peptide conjugated to polymersomal epirubicin enhanced uptake and anti-cancer efficacy of epirubicin against multiple myeloma cells in vivo (Gu et al. 2019). Furthermore, nanoparticles can be decorated with anti-CD44 antibodies. Multifunctionalized iron oxide magnetic nanoparticles with CD44 antibodies were able to selectively target CD44-positive cancer cells (Aires et al. 2016). In breast cancer cells, conjugation of saporin, a ribosome-inactivating protein, with hyaluronan modified nanoparticles achieved intracellular release of saporin leading to enhanced apoptosis (Ding et al. 2018). Hyaluronan-coated nanoparticles have been also conjugated with AS1411 aptamer, to penetrate blood-brain barrier and deliver docetaxel to CD44- overexpressing glioma cells. Delivery of docetaxel through this pathway significantly attenuated the formation of spheroids and tumor growth in vivo (Wang et al. 2019).

18.3.2 Antibodies

Different monoclonal antibodies have been raised against CD44 standard and variant isoforms to be utilized for cancer treatment. One of the best-studied anti-CD44 monoclonal antibodies is RG7356 which binds to the constant region of CD44, abrogating hyaluronan binding. In chronic lymphocytic leukemia (CLL), interruption of hyaluronan/CD44 interaction induced caspase-dependent apoptosis, with the strongest effects being observed in ZAP-70$^+$ CLL cells both in vitro and in vivo (D'arena et al. 2014). Mechanistically, RG7356 engages CD44 to induce its internalization in CLL cells, and subsequently decrease ZAP-70, which was found to be complexed with CD44 (Zhang et al. 2013). RG7356 is also effective against triple-negative breast cancer cells in mice xenografts by modifying the MAPK pathway (Weigand et al. 2012). Response to treatment with RG7356 in xenograft models and colorectal cancer patients depended on the presence of CD44s isoform, suggesting that only treatment in patients with CD44s expression could be effective (Birzele et al. 2015). RG7356 mechanism of function also involves activation of the immune system. Specifically, RG7356 treatment induced secretion of chemo-attractants responsible for recruitment of immune cells, such as macrophages, to the tumor, through activation of MAPK. Moreover, RG7356 activated antibody-dependent cellular phagocytosis (ADCP) of triple-negative breast cancer cells by macrophages (Maisel et al. 2016). In clinical trials, ^{89}Zirconium-labeled RG7356 was taken up by several tissues such as the spleen, liver, bone marrow, lung, and kidney in a dose-dependent manner in patients (Jauw et al. 2018), while selectively targeting CD44$^+$ breast and pancreatic cancer cells in monkeys (Vugts et al. 2014). Moreover, phase

1 clinical trials with RG7356 in patients with advanced, CD44-expressing solid tumors showed that it was well-tolerated, with most side effects being mild such as fever, headache, and fatigue. Its clinical efficacy was modest with 21% of patients experiencing disease stabilization (Menke-Van Der Houven Van Oordt et al. 2016). Furthermore, phase 1 studies in acute myeloid leukemia patients verified the mild adverse effects, although only 2 out of 44 patients showed response in the treatment suggesting that this antibody cannot be utilized as monotherapy (Vey et al. 2016).

Another popular anti-CD44 antibody is A3D8. On human acute myeloid leukemia (AML) cells, A3D8 treatment caused G0/G1 cell cycle arrest through induction of p21, p27 and reduction of pRb and CdK2/4 activities. Furthermore, JNK protein expression was reduced leading to reduction in c-Jun phosphorylation after A3D8 treatment (Zada et al. 2003; Gadhoum et al. 2004a, b; Li et al. 2016). A3D8, apart from inducing apoptosis and inhibiting cell proliferation, induced differentiation of the AML cells (Gadhoum et al. 2004a). This apoptotic effect was caused by A3D8-induced CD44s lipid raft clustering leading to Fas aggregation and subsequent caspase-8 activation (Qian et al. 2012). It is important to note that bone marrow stromal cells could protect acute myeloid leukemia cells from A3D8-induced apoptosis through activation of PI3K/Akt signaling to down-regulate p27 (Chen et al. 2015). In these studies, the effects of A3D8 were verified by another anti-CD44 antibody, H90. In chronic lymphocytic leukemia, A3D8 abrogated CLL cell viability and in vivo caused reduction of MCL1 protein and activation of caspases (Fedorchenko et al. 2013). In human erythroleukemia cells, A3D8 also evoked cell growth inhibition and caspase-independent apoptosis-like cell death, through disruption of mitochondrial membrane potential and release of AIF but not cytochrome c, which is typical for caspase-dependent apoptosis. This type of cell death involves activation of PARP and calpain, since their inhibition ameliorated A3D8-induced cell death (Artus et al. 2006). CD44 ligation by A3D8 was also able to arrest ovarian cancer stem cells in S phase and induced apoptosis (Du et al. 2013).

The monoclonal antibody F77 was developed to recognize glycolipids and O-glycosylation on prostate cancer cell proteins. F77 antigen was finally identified to be glycosylated CD44v10 isoform. On functional level, F77 induced apoptosis in prostate cancer cells. Moreover, this antibody can be utilized in ELISA assays to identify this glycosylated CD44v10 isoform in prostate cancer cells and serum of patients with prostate cancer (Chen et al. 2018b).

Hermes-1 is an anti-CD44-specific monoclonal antibody that specifically interrupts hyaluronan/CD44 interactions. When compared with other anti-CD44 antibodies, like Hermes-3, J173, and 50B4, Hermes-1 was the only one capable to completely inhibit binding of hyaluronan by colorectal carcinoma cells. It is important to note that Hermes-1, Hermes-3, and J173 inhibited the adhesion of the cells on laminin and collagen, while 50B4 did not have any effect (Ishii et al. 1993). Hermes-1 can be also utilized to investigate differences between metastatic cells and cells from the original tumor. Unexpectedly, utilizing Hermes-1, it was found that primary human colon carcinoma cells expressed higher amounts of CD44 on their cell surface, than the cells in lymph node metastases (Kubens and Zanker 1998).

Inhibition of hyaluronan/CD44 interactions has been also achieved with IM7 monoclonal antibody. Despite not displaying any effect on colon carcinoma cell growth in vitro, IM7 was able to inhibit liver metastasis in vivo, although tumor cell colonies were detected in all of the livers even in mice free of nodules, suggesting that IM7 probably delayed formation of metastases, rather than completely inhibiting them (Ogoshi et al. 1998). In glioma cells, which synthesize high levels of hyaluronan, IM7 decreased hyaluronan biosynthesis, and induced apoptosis (Wiranowska et al. 2010). IM7 can be also delivered with chitosan polylactic acid-coated nanoparticles to reduce its toxicity. This modified IM7 antibody was able to reduce proliferation of ovarian cancer cells and control the development and progression of ovarian cancer in vivo (Yang et al. 2017). It is known that hyaluronan fragments that engage CD44 can activate downstream NF-κB signaling in various tumor types, which in turn promotes inflammation and tumorigenesis. IM7 by inhibiting these interactions successfully reduced NF-κB activation in bladder cancer cells (Fitzgerald et al. 2000). IM7 was also able to block adhesion of melanoma cells on endothelial cells, a critical step during metastasis formation (Ota et al. 1995). IM7 can be also conjugated to ribosome inactivating protein saporin, to specifically target CD44-expressing cancer cells. These modified antibodies can release saporin in the target cell cytoplasm, which can then display its cytotoxic effects only on prostate cancer cells with high CD44 expression (Bostad et al. 2014). IM7 also enhanced natural killer cell activity (Tan et al. 1993), suggesting that CD44 targeting with antibodies can enhance activation of immune system against cancer cells. In the same study, different anti-CD44 antibodies were also studied, Hermes-1, S3 and S5, but the same effect was only observed with S5 antibody. The effect of CD44 ligation was also investigated with J173 and F10442 antibodies, where CD44 cross-linking upregulated CD16-mediated lysis. Moreover, CD44 blocking with these antibodies led to rapid increase of intracellular Ca^{2+} (Galandrini et al. 1994). Accordingly, J173 activated MAPK signaling to enhance the killing activity of peripheral mononuclear cells against cancer cells from Burkitt's lymphoma and chronic myelogenous leukemia (Ishizuka et al. 2008).

Several other less studied anti-CD44 monoclonal antibodies have been developed. The HI44α antibody effects were investigated in acute myeloid leukemia cells derived from patients. Ligation of CD44 by HI44α was able to induce differentiation and apoptosis of AML cells, probably through inhibition of c-Myc expression (Song et al. 2004). In colon cancer cells, engagement of CD44 by a specific antibody reversed the resistance to anti-integrin antibody, altered cell morphology, and enhanced apoptosis (Bates et al. 1998). U36 is another antibody that has been raised to recognize CD44v6- expressing squamous cell-carcinomas (Van Hal et al. 1996), but whether it can be used for treatment of these tumors remains to be investigated. KMP1 is a CD44-specific antibody that shows significant anti-tumor effects against bladder cancer cells. Specifically, KMP1 inhibited the bladder cancer cell proliferation, migration, and adhesion in vivo, while suppressed tumor growth in xenograft models. Importantly, expression of the KMP1 epitope correlated with clinical severity and prognosis of bladder cancer (Chen et al. 2018c). Anti-CD44 antibody conjugated to oil liquid nanocapsules, aCD44O^2LNCs, to target pancreatic cancer

stem cells, has also been developed. This technology displayed high uptake of these nanoparticles by pancreatic cancer stem cells in vivo. Moreover, these nanoparticles when coupled with paclitaxel were able to enhance their anti-tumor efficacy (Navarro-Marchal et al. 2021). A defucosylated anti-CD44 antibody, 5-mG2a-f, also significantly reduced tumor development in oral squamous cell carcinoma xenograft models (Takei et al. 2020). In another study, the authors developed four anti-CD44 monoclonal antibodies, namely, P4G9, P3D2, P3A7 and P3G4 that recognized unglycosylated and conserved regions of CD44 ectodomain. P3D2 was able to inhibit breast cancer tumorigenesis in animal models (Lusche et al. 2021). Since CD44v7 is expressed in many human cancers, an antibody raised against CD44v7 is to be utilized as a diagnostic and therapeutic tool in clinical settings (Borgya et al. 1995). Whether this antibody is useful remains to be investigated. An antibody raised against CD44v9, mAb 44-IV was able to inhibit liver metastasis of human colon cancer cells by blocking their adhesion to the capillaries, a critical step in the metastatic cascade (Seki et al. 1997).

18.3.3 Peptides

Apart from antibodies, peptides have been also used to target CD44 and regulate hyaluronan binding capacity. Peptides can be used for detection of CD44-expressing tumor cells inside the tissues. Several peptides against CD44 have been described utilizing phage display libraries (Park et al. 2012a). For example, RP-1, a 12-mer peptide isolated from a phage peptide library, binds to $CD44^+$ gastric cancer cells and allows their detection inside the tissues (Zhang et al. 2015). Importantly, RP1 peptide binding can predict prognosis of gastric cancer patients (Li et al. 2017a). Another 7-mer peptide also isolated from phage display library, termed CV-1, was able to detect CD44v3-v10 protein expression in gastric cancer cells and tissues (Zhang et al. 2016). Similarly, a phage displays 15 amino acid peptide PFT marked CD44v6-expressing prostate cancer stem cells (Peng et al. 2017). Polyvalent-directed peptide polymer (PDPP) specifically traced CD44-expressing breast cancer stem cells (Cho et al. 2015).

A widely studied peptide against CD44 is A6. A6 peptide is derived from uPA, but it does not bind to uPA receptor (uPAR) nor interferes with uPA/uPAR interactions. Importantly A6 did not cause any significant toxicity in animals. Moreover, A6 showed efficacy and exceptionally good safety profile in Phase 1a, 1b and 2 clinical trials. In chronic lymphocytic leukemia, A6 displayed significant toxicity against B-lymphocytes expressing ZAP-70 (Finlayson 2015). A6 peptides loaded with reduction-sensitive polymersomal vincristine sulfate targeted CD44-expressing acute myeloid leukemia cells and reduced the leukemia burden in the circulation, bone marrow, liver, and spleen, while extending survival of mice (Gu et al. 2021). A6 also inhibited migration of ovarian and breast cancer cells. Mechanistically, A6 regulated CD44-mediated adhesion to hyaluronan and the activation of downstream

FAK and MAP/ERK signaling pathways. In vivo treating mice with A6 inhibited the metastasis of melanoma cells to the lung (Piotrowicz et al. 2011).

Peptides that bind CD44v have been also developed. A peptide mimicking a specific extracellular motif of CD44v6 reduced CD44v6-mediated activation of c-Met and VEGFR-2. Treatment with this peptide in vivo reduced angiogenesis in tumors, suggesting that targeting the co-receptor functions of CD44 is another viable possibility (Tremmel et al. 2009). Following the same notion, v6 peptide that interfered with CD44v6 co-receptor functions with these receptors, blocked tumor growth and metastasis in pancreatic tumors in mice, while extended their survival time. Of note, the v6 peptide achieved higher inhibition than c-Met or VEGFR-2 inhibitors (Matzke-Ogi et al. 2016). NLN and NEW are peptides that bind CD44v6, inducing its internalization to inhibit c-Met/Erk pathways, leading to reduction of cell migration, invasion, and metastasis. In vivo these peptides after conjugation with KLA pro-apoptotic peptides successfully killed tumor cells and impaired tumor growth and metastasis without displaying systemic side effects (Khan et al. 2021).

The FK506-binding protein-like (FKBPL) and a 24-amino acid-derived peptide AD-01 exerted anti-angiogenic effects and reduced tumor growth in vivo, effects that are dependent on the presence of CD44 (Valentine et al. 2011). Moreover, FKBPL and AD-01 bound CD44 and reduced breast cancer cell migration, through inhibition of Rac-1 activity, upregulation of RhoA and the actin-interacting proteins profilin and vinculin (Yakkundi et al. 2013). Moreover, AD-01 treatment inhibited mammosphere-forming capacity of breast cancer stem cells in vitro and reduced tumor initiation in vivo. Specifically, AD-01 induced differentiation of breast cancer stem cells, while reducing the expression of stem cell markers Nanog, Oct4, and Sox2. Importantly when combined with DAPT—a Notch inhibitor—it caused significant reduction of chemotherapy and radiotherapy resistance in breast cancer stem cells (McClements et al. 2013).

CD44, apart from binding hyaluronan, can also interact with other proteins like collagen. Consequently, there are also peptides taking advantage of collagen/CD44 interactions. Peptides derived from collagen type IV incorporated to liposomes bearing doxorubicin targeted $CD44^+$ melanoma cells and reduced tumor size (Ndinguri et al. 2012). Moreover, taking advantage of pro-MMP9 and CD44 interactions, P3 and P6 peptide were prepared from PEX9 domain of pro-MMP9, and were shown to reduce chronic lymphocytic leukemia cell adhesion to pro-MMP9 and thus abrogated chemotaxis and transendothelial migration (Ugarte-Berzal et al. 2014). The peptide PCK3145 induced CD44 shedding by increasing MT1-MMP while decreasing MMP9 secretion. PCK3145 also abrogated adhesion of fibrosarcoma cells on hyaluronan, thus antagonizing tumor metastatic processes (Annabi et al. 2005). A5G27 peptides derived from laminin could also bind CD44v3 and CD44v6, and could be conjugated to particles bearing specific siRNAs. Introduction of such particles in vivo inhibited tumor growth by lung adenocarcinoma or ovarian carcinoma cells (Golan et al. 2016). A5G27 peptide blocked melanoma metastasis by inhibiting the binding of FGF2 in the heparan sulfate chains of CD44v3, and thus reducing bioactivity of FGF2 (Hibino et al. 2005). C21, a C-terminal peptide of thrombospondin-4 competed for osteopontin and hyaluronan

binding to CD44, but its effect on tumor cells remains to be investigated (Sadvakassova et al. 2009). Another CD44-binding peptide (CD44BP) added to an engineered matrix showed significant anti-tumorigenic effect, as evidenced by reductions in tumor sphere formation in vitro (Yang et al. 2013).

Another interesting approach was synthesizing CD44 cytoplasmic tail peptides bearing phosphor-Ser^{325} which were conjugated to penetration sequences in order to enhance plasma membrane translocation. Such peptides blocked CD44-mediated cell migration without affecting hyaluronan binding or CD44 expression (Peck and Isacke 1998). CD44 could be used also as an antigen to be recognized by immune cells for cancer treatment. Specifically, CD44-derived peptides could function as immunogens to sensitize dendritic cells and enhance their anti-tumor activities against prostate cancer stem cells that expressed high CD44 levels in vitro and in vivo (Wang et al. 2020).

18.3.4 Chemically Modified Hyaluronan

Hyaluronan can be modified in several different chemical groups to inhibit binding to CD44, affect tissue architecture and to be utilized in hydrogels and as a scaffold for drug carriers. Sulfhydryl (-SH) modified hyaluronan is utilized to form hydrogels to be used as drug-loaded implant for chemotherapeutics, photosensitizer and photothermal reagent in chemotherapy, photodynamic and photothermal therapy against tumors (Xu et al. 2021). Hyaluronan modification with Au-Ag alloy can be used in nanoparticles that allow for the sensitization of breast cancer cells in radiotherapy, since ionizing radiation releases toxic Ag^+ and enhances production of OH^- in tumor sites (Chong et al. 2020). High molecular weight hyaluronan can be also modified with a hydrazide group and bisphosphonate (BP) for selective targeting of CD44 expressing cancer cells (Varghese et al. 2009). Notably, hyaluronan—a non-sulfated glycosaminoglycan—has been modified with sulfate groups which allowed simultaneous targeting of CD44 and P-selectin, to effectively target $CD44^+$P-$selectin^+$ cancer cells (Bhattacharya et al. 2020). Hyaluronan can be also modified with poly(lactic-*co*-glycolic acid) to produce highly stable and GSH sensitive micelles. Loading of these complexes with transferrin-targeted nanoformulated AUY922 eased their uptake by brain tumor cells and induced caspase-dependent cleavage of the apoptosis marker PARP followed by upregulation of p53. Moreover, this complex was drastic against tumor growth in vivo, without displaying any toxicity to other major organs (Debele et al. 2021). β-Cyclodextrin-modified hyaluronan with drug conjugates was able to exert significant toxicity against lung and prostate cancer cells with high expression of CD44, while on the other hand showed no toxicity against normal cells with low CD44 expression (Bai et al. 2020).

18.3.5 Gene Therapies

A promising strategy to target hyaluronan/CD44 network in tumors is to silence the expression of CD44 gene, with specific siRNA and/or shRNA or the most recently developed CRISPR/Cas9 system.

Introduction of a CD44-specific siRNA in lung cancer cells reduced the expression of the stem cell-related genes CXCR4 and POU5F genes after TGF-β1/TNFα treatments but failed to reverse EMT gene signature (Nurwidya et al. 2017). A recombinant adenovirus bearing CD44 shRNA was also able to inhibit cell proliferation, migration and invasion, while induced apoptosis of colon cancer cells. These adenoviral particles inhibited Akt and GSK-3β signaling pathways. Furthermore, the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL were reduced, while the apoptotic proteins Bax, cleaved caspase-3/−9 and PARP were increased in colon cancer cells treated with the adenovirus (Lee et al. 2017). Targeted deletion of CD44v6 with specific shRNA in colon cancer cells also reduced the adenoma growth in vivo through interruption of hyaluronan/CD44v6/pErbB2/Cox-2 pathway (Misra et al. 2009). In triple-negative breast cancer cells, knock-down of CD44 with shRNA vectors significantly suppressed proliferation, colony formation, and invasion (Zhou et al. 2018). Doxorubicin resistance is a major caveat in the treatment of breast cancer. Targeting of breast cancer cells with CD44 siRNA in combination with doxorubicin treatment effectively limited tumor metastasis, proliferation, invasion, migration, and induced apoptosis in triple-negative breast cancer cells, signifying the importance of targeting CD44 to overcome drug resistance (Vahidian et al. 2020). Targeting of CD44-expressing breast cancer cells can be also performed with CD44-targeted aptamer Apt1. This aptamer conjugated to liposomes with encapsulated CD44 siRNA was able to successfully target CD44-expressing breast cancer cells to silence CD44 in vitro and in vivo (Alshaer et al. 2018). In another study, the authors utilized biodegradable poly D,L-lacticied-*co*-glycolide acid nanoparticles (PLGANPs) to deliver FAK and CD44 shRNAs in ovarian cancer cells in vivo, since both FAK and CD44 have active roles in tumor angiogenesis and cancer metastatic processes. Double knock-down of both FAK and CD44 reduced tumor size, inhibited angiogenesis, reduced proliferation and induced apoptosis (Zou et al. 2013).

CRISP/Cas9 technology in contrast to siRNA or shRNA systems offers silencing capacity of a specific gene by completely removing the target gene from the host genome. In osteosarcomas, high expression of CD44 predicts poor survival and higher incidence of metastases in patients. Targeting CD44 with CRISPR/Cas9 system in metastatic osteosarcoma cells abrogated the proliferation and spheroid formation in 3D cultures as well as migration and invasion (Liu et al. 2018). Furthermore, in multi-drug-resistant osteosarcoma cells, silencing of CD44 with CRISPR/Cas9 system could also enhance drug sensitivity (Xiao et al. 2018). Although CD44 seems to play an important role in tumor growth, CD44 silencing in vivo with CRISP/Cas9 in HAS3-overexpressing stromal fibroblasts or CD44 knock-down with shRNA in breast cancer cells was not able to inhibit tumor growth,

in contrast with the reductions that were observed when hyaluronan production or accumulation was inhibited (Zhao et al. 2019). This rather contradicting study points out the context-dependent effects of hyaluronan and CD44 in several tumors. Given the fact that in most breast tumors HAS2 has been most extensively studied, while the role of HAS3 remains obscure, further studies are needed to clarify this discrepancy. CD44 can be also indirectly targeted, as evidenced by CRISPR/Cas9 silencing of Cosmc, an endoplasmic reticulum-localized chaperone that regulates protein O-glycosylation. Cosmc knock-out inhibited protein expression of CD44, confirming the notion that O-glycosylation is important for proper CD44 expression. Of note, reconstitution of CD44 reversed the effects of Cosmc disruption on MAPK signaling and breast cancer cell proliferation, verifying the important role of CD44 for breast tumor growth (Du et al. 2020a). In liver cancer stem cells, knock-out of CD44 utilizing CRISPR/Cas9 system resulted in less malignant and more differentiated tumors. Unexpectedly, CD44 silencing increased the expression of stem cell markers Oct4, Sox2, and Nanog. This contradictory finding warrants further investigation as in these cells CD44 was predominantly nuclear and bound in promoter regions of c-Myc and Sox2 (Han et al. 2015). The CRISPR/Cas9 system can be also utilized to target specific variants of CD44. In gastric cells, CD44 exon v6 was deleted, allowing for the rest of CD44 gene to be expressed. Removal of v6 exon from CD44 sensitized gastric cancer cells to cisplatin and abrogated their self-renewal (Lobo et al. 2020). In another study, utilizing CRISPR/Cas9 and overexpression approaches, chimeric antigen receptors (CARs) that specifically target CD44v6 from head and neck squamous cell carcinoma were expressed on T cells. Targeting CD44v6 demonstrated a direct correlation of CD44v6 expression and cytotoxic effects mediated from CAR T cells (Haist et al. 2021).

18.4 Conclusions

Conclusively, hyaluronan/CD44 network appears to offer an important target for translation into the clinic to treat tumors. However, given the omnipresent localization of hyaluronan in the body and ubiquitous expression of CD44 as well as their importance in physiological processes, targeting this system needs to be approached with caution. On the bright side, many of the hyaluronan effects depend on the expression of CD44v isoforms that are expressed specifically by tumor cells. Moreover, while many of the processes controlled by hyaluronan/CD44 network overlap with features of immune and inflammatory pathways, there are clear differences between tumor cells and physiologic events that can be exploited, like the size and interactions of hyaluronan found in cancerous tissues. Certainly, further research is needed to clarify the exact roles of hyaluronan and CD44 in tumorigenesis, but the evidence presented so far suggests that targeting of this system is a promising avenue for the development of safe and effective cancer treatment regiments in the future.

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