Biology of Extracellular Matrix

Rolf A. Brekken
Dwayne Stupack *Editors*

Extracellular Matrix in Tumor Biology

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Preface

One of the first "mantras" encountered by non-matrix biologists, as they enter the field of matrix biology, is that the extracellular matrix (ECM) is not simply the glue that holds tissues together. To many matrix biologists, this notion is self-evident; after all, biologists have understood for more than eight decades that there is a unique molecular complexity to the ECM and, moreover, have known that different histological tissues exhibit different ECM components. The concept that the complexities and variances in ECM deposition are critical to the unique mechanical and adhesive structures of a given tissue is appreciated and validates the current collection of chapters focused on the contribution of the ECM to cancer biology. Why should it be a surprise that the ECM would be critical to guide, inform, sustain, and signal under essentially every physiologically relevant condition, including cancer?

In a world where we are witnessing an increasing average life span, age-related diseases have greater incidence and impact. Chief among these is cancer. A disease known from the beginnings of medicine, it was assigned its modern name in ancient Greece but remains poorly understood even today. As we develop and implement new approaches to treat cancer, life spans are being extended, yet de facto cures are rare. The disease remains enigmatic, and we are increasingly appreciating that it is a plastic disease, changing as it moves from oncogenesis to late stage. Cancer cells evolve within a patient to adjust to changing selection conditions, whether to escape the homeostatic limits imposed by normal cells, evade immune detection, compete with neighboring tissues and cancer cells for expansion, or resist the challenge of therapy. The ECM is critical for this plasticity and to tumor pathology in general.

A study of the literature over the last two decades can give a reader an indication of the normal function of individual components of the ECM and how they participate in development, wound healing, and tissue homeostasis. We have a good understanding of what the normal functions of ECM components tend to be from human genetic diseases and from mouse models. However, during neoplasia, all normal rules of development and cellular regulation are subject to change. The normal patterns of ECM expression can be altered; the organization of the ECM can

change due to alternative and even rare splicing patterns of ECM components, which may be exposed to proteases not normally encountered during development and homeostasis, or even due to the co-expression of ECM components that are not normally co-expressed within a given tissue. These new and different possible combinations conspire to increase tumor plasticity. Such combinations offer the opportunity to provide new signals or alter the interpretation of normal signals. Such alterations offer new ways for a tumor to sustain itself during critical challenges. Indeed, many of the most famous and impactful oncogenes, such as Ras proteins, P53, and phosphoinositide 3-kinases, are known to be intimately impacted via cell interaction with the ECM. Moreover, receptor tyrosine kinases, which are also key oncogenic drivers, typically depend upon cellular contact with an ECM to function. It is therefore not unexpected that the overexpression of these proteins in cancer, or expression of their oncogenic forms, would be impacted by the very nature of cell contact with the ECM.

This dystopic ECM can ultimately contribute to the pathology of cancer in a number of different ways. Signals received from the ECM can guide tumor cells, encouraging tissue invasion or intravasation and the subsequent local or distant metastases. The distribution and componentry of the ECM guides the relative types of cell migration that are required or advantaged during these processes. These include mesenchymal migration along fibrils of ECM or amoeboid types of movement that proceed by leveraging cell movement through pores in the ECM. Thus, the migration of cancer cells through the brain, or along the bone or along blood vessels, provides unique scenarios that favor cell invasion via different mechanisms. The density of the ECM directs the mechanical and structural support of a tumor, thus dictating how migration is best accomplished. Such density can also influence other determinants of outcome, such as the capacity of chemotherapy to penetrate, and ultimately impact, a tumor. As a result, highly desmoplastic cancers, such as pancreatic cancer, impose special challenges for therapy.

The influence of a dysregulated ECM extends to normal, nonmalignant cells in the tumor microenvironment. The impact of exposure to wound-like ECM components can induce changes in normal tissue that facilitate tumor cell invasion. Further, ECM-induced reprogramming of fibroblasts, selective modulation of immune cell activation, and local metabolic reprogramming can all impact tumor pathology. The precise signaling nature of these events, however, is unique and varies from cell to cell. Recent efforts have begun to quantify and characterize the vast number of ECM proteins in the tumor microenvironment, but equally important will be an understanding of their relative distribution and their interactions with each other and the surface of cancer cells. Understanding these differences is very likely to impact our comprehension of the local drivers of a given cancer.

The recent appreciation of a subpopulation of tumor cells, tumor-initiating cells, and cancer stemlike cells (CSCs) and the concept of tumor dormancy offer new dimensions to our understanding of the tumor ECM. CSCs flourish in a tumor stemlike cell niche that consists in part of a specialized ECM that provides mechanical cues to tumor-initiating cells. Many of the known CSC markers are adhesion receptors. It is easy for matrix biologists to forget or ignore the fact that

markers such as CD49f is α6 integrin or that CD44S is also HCAM (the major receptor for hyaluronan). Matrix biologists interested in cancer biology should use this and many other well-documented examples of how ECM biology affects cancer development and progression to highlight the critical importance of ECM biology in cancer. Matrix biologists need to be the flagbearers for key questions, such as whether the elevated expression of these receptors on the surface is functionally relevant and whether they are in constant use or simply poised to give strong and immediate signals when a permissive environment is encountered.

The goal of this volume is to better integrate our understanding of the contribution of the ECM to tumor progression. While it is not possible to assemble a tome that encompasses all our advances in an exhaustive fashion, this volume represents a survey of recent advances that have significantly added to our concept of the tumor microenvironment and the function of the ECM in it. The chapters cover topics that range from classic matrix components such as fibronectin, to proteoglycans, to proteinases and hybrid molecules that bridge the protease/matrix field. Such molecules, as well as non-protease matricellular proteins that dance in and out of a rigid assembled matrix, all dramatically alter ECM dynamics and function. Indeed, even as we consider tumor heterogeneity, we have to appreciate that this will be accompanied by ECM heterogeneity. Local ECM distribution might be influenced by programmed or stochastic factors. In either case, such alterations can elicit different behaviors, even among genetically similar cells. It is hoped that beyond being a simple aggregation of data and a review of the current state of knowledge, the similarities and differences in signaling listed in these chapters will support the next level of matrix dissection: that of additive and modulating effects. Understanding this complexity will be important to the comprehension of the complexity of the underlying disease.

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Contents

Chapter 1
The Extracellular Matrix of Tumors: A Focus on Fibronectin and Fibulin-5

Mary Topalovski and Rolf A. Brekken

Abstract The extracellular matrix (ECM) in tumors is highly dynamic and contributes to tumor evolution. Fibronectin (FN) is a key component of the ECM in tumors that ligates and stimulates integrins on tumor cells, fibroblasts and endothelial cells in the tumor microenvironment. FN induced integrin activity is reduced by fibulin-5 (Fbln5), a matricellular protein that competes with FN for integrin binding but does not stimulate integrin signaling. A consequence of FN-induced integrin activation is the generation of reactive oxygen species (ROS), which can promote cell survival or apoptosis pending the microenvironment. The tumor microenvironment Fbln5 can be viewed as a molecular rheostat that tunes FN stimulated integrin-induced ROS generation.

1.1 The Function and Composition of the Extracellular Matrix

The extracellular matrix (ECM) is a dynamic collection of secreted molecules, which occupies the space between cells and provides the structural framework necessary to maintain tissue integrity. That structural framework includes proteins such as collagens, fibronectin, laminin, and elastin. Collagens are the most abundant protein in the ECM in the human body. There are multiple types of collagen found within the ECM, and they exist as fibrillar proteins, providing stiffness to the tissue (Vogel 2001). Fibronectins (FN) are glycoproteins that bind to other ECM proteins and help connect cells to the ECM and promote cell movement (Pankov 2002). Laminins are glycoproteins found in the basal laminae of all cell types, where they form weblike networks that provide tensile strength to the tissue (Beck et al. 1990). Finally, elastins are proteins that provide flexibility to the tissue, allowing the tissue to retain shape after stretching or contraction (Mithieux and Weiss 2005).

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Many diverse cell types can secrete ECM molecules; however, tissue fibroblasts are typically the source of the majority of ECM synthesis and secretion.

The most common and well-studied functions of the ECM are cell adhesion and cell-to-cell communication. ECM proteins are anchored to the cell through cell surface receptors, the most intensively studied of which are integrins. Integrins are heterodimeric transmembrane receptors consisting of α and β subunits. In mammals, there are at least 18 α subunits and 8 β subunits, generating 24 unique integrins (Harburger and Calderwood 2009), which have ligand specificity and, as a result, distinct functions. For example, α5β1 (a major FN receptor) is important during angiogenesis (Kim et al. 2000), whereas α1β1 and α2β1 (major collagen receptors) are critical for leukocyte adhesion and inflammation (de Fougerolles et al. 2000; Krieglstein et al. 2002).

In addition to providing a structural framework for tissues, the ECM also regulates a myriad of important signaling pathways. As integrins are ligated, they form clusters at the membrane, referred to as focal adhesions. Focal adhesion complexes are essential to induce downstream signaling, as the cytoplasmic tails of integrins do not harbor any detectable enzymatic activity (Harburger and Calderwood 2009). Integrin clustering results in the reorganization of the actin cytoskeleton and the activation of downstream signaling complexes. Integrins are unique in that they relay signals from inside the cell to the outside (inside-out signaling) and vice versa (outside-in signaling) (Giancotti 1999; Topalovski and Brekken 2015). Integrin contact with the ECM results in outside-in signaling, whereas protein complexes from inside the cell bind to integrin tails, which is thought to prime integrins for ECM interaction (inside-out signaling).

Integrin clustering and focal adhesion assembly activate protein tyrosine kinases such as focal adhesion kinase (FAK) (Lipfert et al. 1992). FAK was one of the first phosphorylation targets discovered downstream of integrin activation (Masur et al. 1995). An immediate downstream target of FAK activity is the major protein kinase Src, which promotes survival, proliferation, and migration (Schlaepfer et al. 1994). FAK is a central signaling scaffold that activates downstream signaling pathways, specifically the Rho-family GTPases, which are critical for actin cytoskeletal dynamics and cell movement. Furthermore, the activation of integrins also promotes growth factor receptor activation. For example, optimum cell stimulation with epidermal growth factor (EGF) requires integrin-mediated cell adhesion (Eliceiri 2001). Therefore, integrins regulate a range of important biological processes from cell migration to cell growth and proliferation.

1.2 The Role of the ECM in Tumor Development with Emphasis on FN

The ECM is also critical in the formation and maintenance of solid tumors. Most solid tumors display increased deposition of ECM proteins and ECM remodeling compared to their normal tissue counterparts. The ECM affects tumorigenic

processes and functions, including tumor cell proliferation, survival, apoptosis, migration, adhesion, angiogenesis, and chemoresistance (Topalovski and Brekken 2015; Aguilera et al. 2014; Akiyama et al. 1995; Bachem et al. 2005; Han et al. 2006; Itano et al. 2008; Lu et al. 2012; Miyamoto et al. 2004). Desmoplasia, the robust deposition of ECM, is induced in tumors by growth factors, such as transforming growth factor β (TGF-β), basic fibroblast growth factor, connective tissue growth factor, interleukin-1β (IL-1β), and platelet-derived growth factor (Hocevar et al. 1999; Leask and Abraham 2004; Bonner 2004; Zhao et al. 2012), and environmental conditions including hypoxia (Aguilera et al. 2014). The ECM is a major facet of the tumor microenvironment (TME), a collective term referring to the immediate environment surrounding tumor cells. In addition to ECM proteins, the TME contains multiple types of non-cancer cells such as immune cells, fibroblasts, and endothelial cells that facilitate tumor progression (Weis and Cheresh 2011; Mao et al. 2013; Gajewski et al. 2013).

In normal tissue, cells remain anchored to their surrounding ECM; detachment from this supportive matrix results in a form of cell death termed anoikis (Frisch and Screaton 2001). However, tumor cells have evolved to circumvent this anchorage dependence leading to the presence of circulating tumor cells that can metastasize to distant organs (Guadamillas et al. 2011). When metastatic tumor cells reach their new site, they must create a microenvironment conducive for survival and growth. Recent work in a mouse model of pancreatic ductal adenocarcinoma (PDA) shows that FN is critical in supporting the engraftment of metastatic cancer cells in target organs (e.g., the liver and lung) (Costa-Silva et al. 2015).

The expression of FN is elevated in many solid tumors, especially PDA (Ramakrishnan et al. 2006; Ramaswamy et al. 2003; Stenman and Vaheri 1981). In this context, FN contributes to cancer cell survival, invasion, metastasis, chemoresistance, and angiogenesis (Topalovski and Brekken 2015). For instance, human PDA cell lines Panc-1 and Capan-1 showed increased resistance to cytotoxic agents including gemcitabine, cisplatin, and doxorubicin when grown on FN-coated plates (Miyamoto et al. 2004). The pro-survival effect of FN has been attributed to the activation of the PI3K/AKT/mTOR pathway (Han et al. 2006; Chen and Guan 1994). Moreover, it was reported that FN stimulates reactive oxygen species (ROS) in PDA cells, which led to increased survival that could be reversed by antioxidant treatment (Edderkaoui et al. 2005). ROS, in moderate amounts, serve as signaling molecules that stimulate proliferation through the Ras-Raf-MEK-ERK pathway and promote survival through the NF-κB pathway (Reuter et al. 2010).

FN is also an important contributor to angiogenesis, the formation of new blood vessels from preexisting vessels. In addition to growth factors such as vascular endothelial growth factor (VEGF), ligation of the ECM via integrin receptors is essential for angiogenesis. Genetic deletion of integrins β1, αv, α4, or α5 in mice individually completely hindered angiogenesis (Schaffner et al. 2013). Integrin α5 knockout mice die at embryonic day E10.5 due to severe vascular defects (Yang et al. 1993), and the deletion of FN results in vascular abnormalities and death at day E9.5 (George et al. 1993). Tumors require angiogenesis to persist and thrive. Although still under active investigation, FN has been shown to be important in tumor angiogenesis as well. Early studies revealed that targeting the FN-α5β1

interaction via antibody antagonists blocked angiogenesis in the chick chorioallantoic membrane assay (Kim et al. 2000). Moreover, α5β1-targeting antibodies, such as volociximab, inhibit tumor growth in various animal models of cancer, but these promising preclinical results have yet to be recapitulated in patients (Ramakrishnan et al. 2006; Besse et al. 2012; Bhaskar et al. 2008; Bhaskar et al. 2007; Cranmer et al. 2005; Kuwada 2007; Ricart 2008).

At the molecular level, FN may stimulate angiogenesis by various mechanisms. Integrin adhesion to the ECM, and to FN specifically, triggers endothelial cell migration and microvessel elongation (Nicosia et al. 1993). Furthermore, the alternatively spliced extra domain A of FN can stimulate VEGF-C expression in a PI3K/Akt-dependent manner in colorectal carcinoma (Xiang et al. 2012). Along these lines, FN can stimulate ROS production (Edderkaoui et al. 2005; Wang et al. 2015; Chiarugi et al. 2003), mainly in the form of hydrogen peroxide, which is known to induce VEGF expression when present in moderation (Zhu et al. 2002).

1.3 Matricellular Proteins: Extracellular Modulators of the ECM

Integrin activation by abundant ECM proteins such as FN and collagen is controlled by regulatory proteins found within the ECM referred to as matricellular proteins. Matricellular proteins as a class do not function as structural components of the ECM; instead, they mediate ECM-receptor interactions (Bornstein and Sage 2002; Wong and Rustgi 2013). Some major examples of matricellular proteins are thrombospondin-1 and thrombospondin-2 (TSP-1 and TSP-2), tenascin-C (TN-C), secreted protein acidic and rich in cysteine (SPARC), osteopontin (OPN), and the fibulin family of proteins. Matricellular proteins can regulate ECM function by directly binding to cell surface receptors or structural and soluble proteins within the ECM. For example, SPARC binds directly to collagen and blocks collagen-mediated signaling via the discoidin domain receptors (Aguilera et al. 2014; Arnold et al. 2010). In this context, the absence of SPARC has enhanced collagen-mediated tumor progression. Moreover, TSP-1 blocks FN-induced focal adhesions in endothelial cells (Murphy-Ullrich et al. 1993). Similarly, TN-C interferes with FN-integrin interaction and reduces the FN-mediated adhesion of fibroblasts (Chiquet-Ehrismann et al. 1988; Huang et al. 2001).

Matricellular proteins are typically expressed abundantly during development and reactivated during wound healing and other tissue remodeling events (Bornstein and Sage 2002). Phenotypes of mice lacking a particular matricellular protein are usually mild, reinforcing the fact that these proteins do not contribute to the structural integrity of tissues (Chapman et al. 2009; Nakamura et al. 2002). However, deficiencies in response to wound healing and tissue repair are often seen (Kyriakides and Bornstein 2003). The abnormal expression of matricellular proteins is seen in certain pathologies, such as cancer. The matricellular protein fibulin-

5 (Fbln5) is aberrantly expressed in many cancers (Wang et al. 2015; Hwang et al. 2013; Lee et al. 2008; Shi et al. 2014; Tang 2015). Fbln5 has important contributions to normal physiology and development as well as cancer.

1.4 Fibulin-5

Fbln5 is a 66 kDa matricellular glycoprotein. It is a member of the fibulin family of ECM proteins, which are characterized by six calcium-binding EGF-like repeats (cbEGF) at the N-terminus (for protein stability and protein interaction) and a globular C-terminal fibulin module (Yanagisawa et al. 2009). Similar to other ECM proteins, Fbln5 is a TGF-β- and hypoxia-inducible gene (Kuang et al. 2006; Guadall et al. 2011). Fbln5 is distinct among the fibulins in that it contains an RGD-integrin binding domain (Fig. 1.1).

Fbln5 was discovered as a gene highly expressed in large vessels during development; however, it is downregulated in most adult tissues except where active tissue remodeling is occurring (Nakamura et al. 1999, 2002; Kowal et al. 1999). Fbln5 is induced by models of vascular injury including balloon withdrawal injury and in atherosclerotic plaques, highlighting its contribution to vascular function and maintenance (Kowal et al. 1999; Nakamura et al. 1999). The generating of Fbln5-deficient mice revealed a major function of Fbln5 in elastic fiber formation (Nakamura et al. 2002; Yanagisawa et al. 2002). *Fbln5*$^{-/-}$ mice exhibited general connective tissue defects such as loose skin, tortuous vessels, emphysematous lung, and genital prolapse. This provided the first animal model for congenital elastic

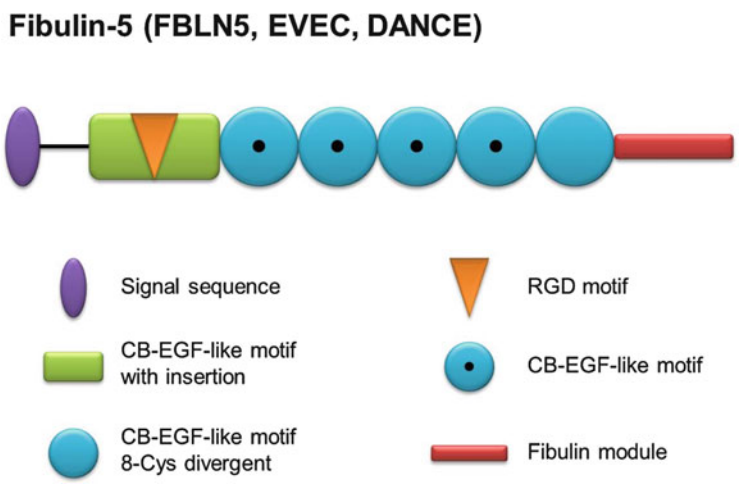

Fig. 1.1 Domain structure of Fbln5. cbEGF (calcium-binding EGF-like domains), RGD (Arg-Gly-Asp; tripeptide binding site of integrins)

fiber disorders (Loeys et al. 2002). These seminal studies laid the foundation for future discoveries regarding Fbln5.

1.4.1 Fbln5 in Cell Adhesion/Migration

As mentioned above, Fbln5 contains an RGD-integrin binding domain and has been reported to ligate RGD-binding integrins including α4β1, α5β1, αvβ3, αvβ5, and α9β1 (Nakamura et al. 2002; Lomas et al. 2007). Fbln5 supports the adhesion of endothelial cells and smooth muscle cells (SMCs) in an RGD-dependent manner (Kowal et al. 1999; Nakamura et al. 1999; Lomas et al. 2007). Furthermore, Fbln5 interacts with multiple extracellular proteins, including extracellular superoxide dismutase (ecSOD) where it tethers ecSOD to endothelial cells where it reduces ROS-induced damage (Nguyen et al. 2004). Fbln5 also binds to tropoelastin and lysyl oxidase-like 1 (LOXL1), which is thought to be critical for normal elastogenesis to occur (Liu et al. 2004).

Fbln5 regulates cell migration in a context-dependent manner. For example, Fbln5 failed to induce migration in SMCs (Lomas et al. 2007). SMCs plated onto Fbln5-coated dishes appeared rounded and less spread compared to cells plated on FN. Immunofluorescence staining showed that focal adhesion and actin stress fiber formation were significantly reduced in the presence of Fbln5 compared to FN. Finally, cells plated on either Fbln5 or FN were analyzed for migratory capacity by imposing a wound in the adherent cultures. Closure of the wound was monitored between the two conditions, which revealed reduced migration in cells plated on Fbln5 (Lomas et al. 2007). Treatment with an activating β1 integrin antibody reversed the negative effects of Fbln5 on migration and stress fiber formation. These results suggest passive ligation of integrins by Fbln5 in SMCs.

Fbln5 also antagonizes endothelial cell migration (Albig and Schiemann 2004). In this context, ectopic expression of Fbln5 in endothelial cells can dampen cell migration as tested by the ability of these cells to move through a Matrigel matrix and also by measuring trans-well migration (in a Boyden chamber assay). Moreover, treating endothelial cells with recombinant Fbln5 blocked angiogenic sprouting in vitro (Albig and Schiemann 2004). In contrast, Fbln5 induced the migration of fibrosarcoma cells (Schiemann 2002). Using a slightly modified version of the Boyden chamber assay where FN was coated on the bottom of the porous membrane, Fbln5-expressing fibrosarcoma cells displayed enhanced migration toward FN, suggesting that Fbln5 promotes a de-adhesive state that facilitates cell migration in certain contexts. These findings highlight cell-type specific effects of Fbln5 signaling that likely reflect the variable integrin expression profile between cell types and whether these specific integrins are subject to inhibition by Fbln5.

1.4.2 *Fbln5 in Proliferation/Survival*

Similar to the context-specific effects on cell motility, Fbln5 can positively and negatively regulate cell proliferation depending on cell type. For instance, the overexpression of Fbln5 in 3T3 fibroblasts revealed the increased activation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) (Schiemann 2002). This group also showed that Fbln5 synergized with TGF-β to stimulate proliferation and DNA synthesis in 3T3 cells. Conversely, Fbln5 was shown to induce an antiproliferative response in epithelial cells through the decreased expression of cyclin A, thus abrogating the progression of the cell cycle.

Endothelial cells stimulated with Fbln5 did not show activation of ERK1/2 and p38-MAPK, but Fbln5 did reduce VEGF expression, which resulted in reduced endothelial cell proliferation (Albig and Schiemann 2004). Likewise, Fbln5 failed to induce proliferation in SMCs compared to cells plated on FN due to a reduction in β1 integrin signaling (Lomas et al. 2007). In addition, recent studies performed in ovarian cancer cells also revealed changes in proliferation upon Fbln5 overexpression (Heo et al. 2015). The expression of Fbln5 in SKOV3 ovarian carcinoma cells led to G2/M arrest but did not adversely affect colony-forming ability. Conversely, knockdown of Fbln5 in human gastric cancer cells blocked cell proliferation as tested by BrdU incorporation (Shi et al. 2014). Together, these data support the presence of an antiproliferative effect of Fbln5 in endothelial cells and a pro-proliferative effect in fibroblasts. Yet, it is unclear whether Fbln5 promotes or blocks proliferation in epithelial cells. Since Fbln5 blocks FN-mediated integrin signaling, it is germane to evaluate the effect of integrin-activating or integrin-inhibiting antibodies on proliferation in contexts where Fbln5 has been manipulated.

1.4.3 *Fbln5 in Angiogenesis*

Fbln5 was identified by two separate groups who were interested in proteins that regulate cardiovascular development and disease (Kowal et al. 1999; Nakamura et al. 1999). Each group identified Fbln5 as being expressed in the embryonic arterial vasculature; however, it was downregulated in most adult vascular beds. They also found that the expression of Fbln5 was reactivated in diseased adult vasculature, namely, atherosclerotic and balloon-injured arteries. *Fbln5*$^{-/-}$ mice were created shortly after these original findings, which revealed the important function of Fbln5 in elastogenesis, in that Fbln5-deficient animals had tortuous vessels in addition to loose skin and emphysematous lungs as a result of incomplete elastic fiber formation (Nakamura et al. 2002; Yanagisawa et al. 2002). Elasticity is a major characteristic of blood vessels and critical for proper vessel function. These observations led to the investigation of Fbln5 as a regulator of angiogenesis in several research models.

Sullivan et al. (Sullivan et al. 2007) revealed that polyvinyl alcohol sponges implanted into *Fbln5*$^{-/-}$ mice had significantly increased vascular invasion as seen by CD31 (PECAM-1) staining. Interestingly, fibroblast migration into these sponges remained unchanged in the absence of Fbln5. This group then examined a possible mechanism by which Fbln5 was antagonizing vascularization and found that the pro-angiogenic factors, Ang-1, Ang-2, Ang-3, and VEGF, were increased in sponges removed from *Fbln5*$^{-/-}$ mice as seen by quantitative PCR. Moreover, vascular smooth muscle cells isolated from WT- and Fbln5-deficient mice also showed an increase of these pro-angiogenic factors in the absence of Fbln5. The authors of this paper did not examine the function of integrins in this phenotype. Therefore, based on these studies alone, it is difficult to determine whether Fbln5 directly antagonizes vascular function or if this is an integrin-dependent phenomenon. Given the evidence that Fbln5 binds to but does not support the activation of α4β1 and α5β1 integrins in SMCs (Lomas et al. 2007), it is probable that enhanced integrin activation in the absence of Fbln5 results in increased endothelial cell migration and proliferation in this context (Lamalice et al. 2007; Clark et al. 1982).

Investigating the function of Fbln5 in tumor angiogenesis has also produced mixed results. The forced expression of Fbln5 by fibrosarcoma cells resulted in tumors that were significantly less vascularized compared to tumors derived from control cells (Albig 2006). These results suggest that Fbln5 antagonizes tumor angiogenesis, which again is likely a consequence of reduced FN-integrin signaling by Fbln5. In contrast, we have reported that subcutaneous and orthotopic pancreatic tumor growth (Pan02) in *Fbln5*$^{-/-}$ mice results in reduced tumor blood vessel density (Schluterman et al. 2010). In this study, the loss of Fbln5 in tumors resulted in elevated ROS production that was mediated by FN-integrin signaling. The discrepancy between these two findings is likely attributed to the complete lack of host Fbln5 in the knockout model as well as the lack of Fbln5 expression in Pan02 cells (Wang et al. 2015; Schluterman et al. 2010). In the fibrosarcoma model, it is difficult to distinguish between the function of host- or tumor-derived Fbln5 with regard to the regulation of angiogenesis. Moreover, the loss of Fbln5 function in tumors produces a much different effect on angiogenesis compared to non-tumor tissue (e.g., the polyvinyl alcohol sponge model mentioned above), which may be due to the high amount of FN in tumors versus normal tissue. A major biological outcome of FN-integrin signaling is ROS production (Chiarugi et al. 2003); therefore, Fbln5 functions as a molecular rheostat to control integrin-induced ROS generation.

1.4.4 Fbln5 in Tumor Progression

As described above, the cellular effects of Fbln5 are context dependent, and thus the effect of Fbln5 on tumor progression may also be tumor specific. Initial studies on Fbln5 and tumor growth relied on the forced expression of Fbln5 in cancer cell lines that were then implanted into immunodeficient mice. Lee et al. (2008) revealed

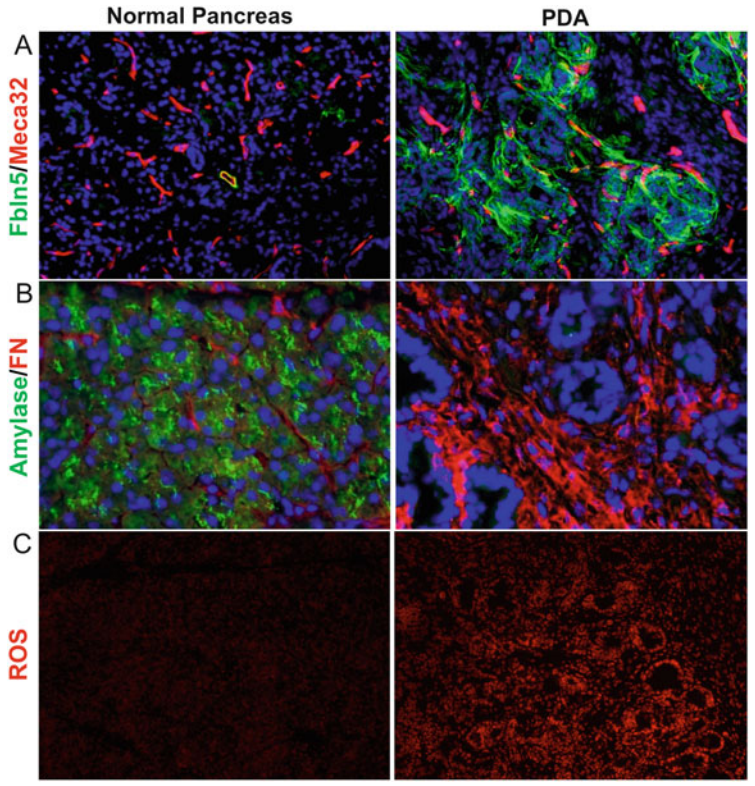

Fig. 1.2 Enhanced ECM and ROS production in mouse PDA. (**a**) Normal (left column) and tumor (right column) pancreas from WT or $Kras^{LSL\text{-}G12D/+}$; $Cdkn2a^{f/f}$; $p48^{Cre/+}$ (PDA) mice stained for Fbln5 (*green*) and Meca32 (*red*), which marks blood vessels. Nuclei were stained with DAPI (*blue*). (**b**) Tissues were stained with amylase (*green*), which stains normal pancreatic acinar tissue, and fibronectin (FN, *red*). (**c**) Tissues were stained with dihydroethidium, a ROS-sensitive chemical red dye. Reproduced from Wang et al. (2015)

increased Fbln5 expression from human breast tumors, and that Fbln5 expression in 4T1 breast cancer cells enhanced tumor growth in mice. Yue et al. (2009) reported that Fbln5-expressing H460 lung cancer cells displayed decreased metastasis to the lungs after IV injection. This study did not examine primary lung tumor growth in the context of Fbln5; however, lung cancer patient samples showed a downregulation of Fbln5 compared to matched normal lung specimens as seen by RT-PCR and immunohistochemistry (IHC) analysis of a tissue microarray.

Fbln5 levels are markedly increased in mouse (Fig. 1.2a) and human PDA (Wang et al. 2015) compared to levels in a normal pancreas. An analysis of various cell types in vitro as well as IHC in PDA samples revealed that stromal cells such as endothelial cells and fibroblasts are the main producers of Fbln5 protein (Wang et al. 2015). Further studies into the factors that control Fbln5 expression in PDA have revealed that the hypoxic TME of PDA induces Fbln5 expression through a

TGF-β-PI3K/AKT signaling axis. The inhibition of a TGF-β receptor (TGF-βR), PI3K, or protein kinase B (AKT) was found to block hypoxia-induced Fbln5 expression in vitro. In genetically engineered mouse models (GEMMs) of PDA, therapy-induced hypoxia (via anti-VEGF treatment) elevated Fbln5 expression, while pharmacologic inhibition of TGF-β signaling reduced its expression.

As mentioned previously, Schluterman et al. (2010) showed that subcutaneous and orthotopic Pan02 tumors implanted into *Fbln5*$^{-/-}$ mice had a significant reduction in tumor growth and blood vessel density (Schluterman et al. 2010). Fbln5 regulates ROS production in vascular tissue through binding ecSOD, and the deletion of Fbln5 results in increased ROS production in mouse aortas (Nguyen et al. 2004). Elevated ROS production can be detrimental to vascular function, and consistent with this notion, Schluterman et al. (2010) found elevated levels of ROS production in tumors grown in *Fbln5*$^{-/-}$ mice compared to tumors grown in *WT* mice. Moreover, mice containing a point mutation in the RGD-integrin binding domain of Fbln5 (RGD → RGE) recapitulated the phenotype seen in *Fbln5*$^{-/-}$ mice with regard to tumor growth, angiogenesis, and ROS production, highlighting the importance of Fbln5-integrin interaction in this phenotype. Using mouse embryonic fibroblasts in vitro, it was shown that increased ROS production is a consequence of increased integrin signaling by FN in the absence of functional Fbln5. Furthermore, antioxidant treatment restored tumor growth and microvessel density in *Fbln5*$^{RGE/RGE}$ mice, confirming that reduced tumor growth and angiogenesis was a direct consequence of elevated ROS production driven by the loss of Fbln5-integrin binding. Expanding on this work, Wang and Topalovski et al. (Wang et al. 2015) found that the mutation of the RGE-integrin binding domain of Fbln5 in the context of a GEMM of PDA also resulted in reduced tumor growth and angiogenesis due to increased ROS production. These data are in line with other evidence that shows Fbln5 competes with FN to negatively regulate β1 integrin function (Lomas et al. 2007).

Elevated oxidative stress is seen in many solid tumors compared to normal tissues, and exploiting this biochemical difference has the potential to enhance the efficacy of anticancer agents (Trachootham et al. 2009). For instance, ROS accumulation after gemcitabine treatment in PDA cells contributes significantly to the cytotoxic activity of this nucleoside analog (Ju et al. 2015). Furthermore, elevated hydrogen peroxide levels were shown to be a mechanism by which paclitaxel killed lung cancer cells (Alexandre et al. 2006). The homeostasis of ROS is important for normal cell function and signaling, but excessive ROS can result in cellular toxicity, and therefore ROS levels must be tightly controlled. ROS can be produced by a number of enzymes, including but not limited to, the electron transport chain (ETC), NADPH oxidase (NOX), 5-lipoxygenase (5-LOX), and nitric oxide synthase (NOS) (Holmstrom and Finkel 2014).

ECM proteins can stimulate the activation of these enzymes and thus indirectly induce ROS production. Chiarugi et al. (Chiarugi et al. 2003) showed that integrin activation by FN induces ROS production in a 5-LOX- and NOX-dependent manner in 3T3 fibroblasts (Chiarugi et al. 2003). Another research group showed that PDA cells are responsive to FN in terms of increased ROS production by 5-LOX and NOX (Edderkaoui et al. 2005). Given that FN can stimulate ROS

production via integrin activation and that the loss or mutation of Fbln5 results in higher ROS production, we propose that Fbln5 blocks integrin-induced ROS production. In PDA, where ECM proteins such as FN are abundant (Fig. 1.2b; Bachem et al. 2005; Mahadevan and Von Hoff 2007), oxidative stress levels are much higher compared to those in the normal pancreas (Fig. 1.2c; Blum and Kloog 2014). Thus, Fbln5 is a novel target to investigate as a potential strategy to manipulate ROS levels in the TME.

1.5 Conclusion

As described in this chapter, there are multiple facets of the ECM that contribute to overall tumor progression and response to therapy. The ECM is highly dynamic, and its contribution to disease progression relies on several structural and nonstructural proteins. Nonstructural matricellular proteins, such as Fbln5, can have pro- and anti-tumorigenic activity, which is highly dependent on the nature and composition of the surrounding TME. The large portion of Fbln5 present in the TME is derived from stromal cells; however, it is unclear whether Fbln5 also exerts its effects on nearby tumor cells. It is likely that the expression profile of integrins on the cell will dictate its response to Fbln5 signaling. In PDA, the loss of Fbln5-integrin binding leads to increased FN-integrin signaling, which counterintuitively results in smaller tumors. This is a prime example that highlights the context-specific effects of ECM signaling on tumor growth. Due to the extremely high levels of FN in PDA, the tumor hijacks Fbln5 to protect itself from the harmful by-product of enhanced FN-induced ROS generation. In this situation, too much FN signaling is unfavorable for the tumor, and thus Fbln5 promotes tumor growth by limiting FN-induced ROS production. Given this evidence, multi-faceted approaches to target the ECM while simultaneously augmenting ROS production in the TME may be a viable and effective therapeutic strategy to combat solid tumors such as PDA.

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Chapter 2
Internalization of Collagen: An Important Matrix Turnover Pathway in Cancer

Dorota Ewa Kuczek, Mie Linder Hübbe, and Daniel Hargbøl Madsen

Abstract Invasive tumor growth is associated with extensive remodeling of the surrounding extracellular matrix (ECM). Degradation of the original ECM scaffolds, which primarily consist of collagens, is key to the morbidity of the cancer as it leads to destruction of the original tissue and replacement with cancerous tissue. The degradation of collagen involves the cleavage of collagen fibers by extracellular proteases and the subsequent receptor-mediated internalization of large collagen fragments for lysosomal degradation. The extracellular cleavage reactions are typically mediated by matrix metalloproteinases (MMPs) that cleave the collagen strands at specific sites, thereby releasing defined collagen fragments. The intracellular collagen degradation pathway is primarily mediated by two endocytic receptors, uPARAP/Endo180 and the mannose receptor (MR), that bind collagen fragments at the cell surface and direct them to the lysosomes for complete proteolytic degradation. Macrophages and most mesenchymal cells can internalize collagen through the action of MR and uPARAP/Endo180, respectively. These receptors bind preferentially to cleaved collagen and cooperate with MMPs to degrade collagen fibers in a sequential process involving MMP-mediated collagen cleavage followed by receptor-mediated internalization of collagen fragments for lysosomal degradation. In vivo, MR-mediated collagen uptake has been suggested to be dominant in many situations with uPARAP/Endo180 playing a smaller role. In connection to cancer, uPARAP/Endo180 is often upregulated and mostly restricted to cancer-associated fibroblasts, and the action of uPARAP/Endo180 promotes tumor growth and counteracts the development of fibrosis. MR is expressed by M2 macrophages in healthy dermis and in solid tumors and mediates the efficient

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internalization of collagen. However, the direct functional consequences of this MR-mediated collagen degradation for cancer growth and invasion still need to be investigated.

2.1 Collagen Degradation in Cancer

During the invasive growth of solid tumors, extensive remodeling of the surrounding extracellular matrix (ECM) occurs (Bonnans et al. 2014). The original ECM scaffolds, which primarily consist of collagens, are degraded, and this contributes to the destruction of the original tissue and replacement with cancerous tissue. The degradation of the ECM furthermore releases pro-tumorigenic growth factors embedded in the matrix (Kessenbrock et al. 2010), and the degradation of the basement membrane, a specialized sheetlike ECM structure, is considered a prerequisite for cancer invasion and metastasis to take place (Rowe and Weiss 2008). Within the last decade, several seminal papers have revealed that another critical consequence of the degradation of the original ECM follows from the fact that this process is accompanied by the excessive deposition of new matrix proteins (Cox and Erler 2011; Lu et al. 2012). These dynamics result in the generation of a novel ECM, which can have a very different composition, organization, and stiffness (Schedin and Keely 2011; Cox and Erler 2014; Bonnans et al. 2014). Often the deposited tumor-specific ECM is of increased stiffness and rich in collagen type I and sometimes also includes variants of matrix proteins that are not typically observed in adults (Schedin and Keely 2011; Cox and Erler 2011; Giblin and Midwood 2015). The altered tumor-specific ECM critically influences the biology of the tumor to ultimately promote tumor growth and metastasis (Joyce and Pollard 2009; Pyonteck et al. 2013; Pickup et al. 2014; Nagelkerke et al. 2015).

In connection to breast cancer, the ECM stiffness and orientation of the collagen fibers critically affect the prognosis of the patients (Conklin et al. 2011; Bredfeldt et al. 2014; Acerbi et al. 2015). The ECM stiffness is primarily determined by the density of deposited collagen and the degree of cross-linking mediated mainly by the enzyme LOX (Levental et al. 2009; Cox and Erler 2011). Furthermore, the density of normal breast tissue correlates strongly with the risk for development of breast cancer (Guo et al. 2001; Boyd et al. 2007). Interestingly, breast epithelial cells respond to increased ECM stiffness in vitro by losing polarity, by proliferating more, and by acquiring a phenotype in many ways resembling malignant transformation (Paszek et al. 2005; Levental et al. 2009).

Collagens are the main protein components of the ECM, although the ECM encompasses many other components including fibronectin, elastin, laminin, entactin, and proteoglycans (Lu et al. 2012). Collagen type I is the main fibrillar collagen of the ECM, and extremely large collagen type I deposits can be observed in connection to cancer (Provenzano et al. 2008; Lu et al. 2012). Collagen type IV is another critical collagen as it together with laminin is the predominant constituent of the basement membrane (Rowe and Weiss 2008). Only a limited number of

secreted or cell membrane-anchored proteases can cleave ECM proteins, and the majority of these proteases belong to the matrix metalloproteinase (MMP) family (Kessenbrock et al. 2010). The activity of MMPs is usually low in tissues under steady-state conditions but increases dramatically under inflammatory conditions including cancer (Egeblad and Werb 2002; Madsen and Bugge 2015). The functional implications of MMPs in cancer have consequently been studied extensively, but broad-spectrum MMP inhibition for cancer treatment has, however, failed to show any effect on survival of the patients in clinical trials (reviewed by Coussens et al. 2002). The reason for this failure most likely relates to the fact that MMP functions are much more diverse than originally believed and not only pro-tumorigenic (Martin and Matrisian 2007; López-Otín et al. 2009; Decock et al. 2011). In addition to remodeling of the ECM, MMPs can also initiate signaling by releasing or activating growth factors (Egeblad and Werb 2002; Kessenbrock et al. 2010) or prevent downstream signaling processes by shedding signaling receptors from the cell surface (Sanderson et al. 2006; Atapattu et al. 2014). Furthermore, the understanding of the role of ECM in cancer progression has increased tremendously over the last decade, and we now know that the ECM is not just an inert physical barrier that needs to be degraded for the cancer cells to invade the surrounding tissue. Rather it provides the tumor cells with multiple cues that affect the biology and function of cancer cells, stromal cells, and infiltrating immune cells (Lu et al. 2011; Salmon et al. 2012; Pickup et al. 2014).

2.2 Extracellular Collagen Degradation

Collagen type I is the most abundant type of collagen and it is the main protein constituent of interstitial ECM. The extracellular cleavage of collagen type I is primarily mediated by MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14 (Krane and Inada 2008; Fields 2013). In the specific case of osteoclast-mediated collagen degradation, the cysteine protease cathepsin K is responsible for the degradation that takes place in low-pH resorption pits formed between the bone surface and the cell (Saftig et al. 1998). MMP-mediated cleavage of collagen type I occurs at defined sites on the polypeptide chains, and the triple-helical collagen strands are especially susceptible to proteolytic cleavage after residue 775 of the α1- and α2-chain of type I collagen (Highberger et al. 1979; Fields 2013). This cleavage reaction results in the release of a large three quarter collagen fragment also designated TC^A, and a small one quarter collagen fragment also designated TC^B. The released one quarter and three quarter collagen fragments partially denature at physiological temperatures making the fragments susceptible to cleavage by other proteases such as the secreted gelatinases MMP-2 and MMP-9 (Hipps et al. 1991) and perhaps also the membrane-anchored serine protease fibroblast activation protein (FAP) (Park et al. 1999; Fan et al. 2015). The detailed molecular mechanisms of MMP-mediated cleavage of collagen have been thoroughly reviewed (Fields 2013). MMPs are upregulated in most solid cancers where they contribute

to the cancer-associated collagen degradation (Egeblad and Werb 2002). The MMP-mediated remodeling of interstitial collagen is an essential part of invasive tumor growth, and degradation of collagen type IV, which is the main component of the basement membrane, is essential for the metastatic spread of the cancer (Tryggvason et al. 1987; Rowe and Weiss 2008). Both cancer cells and stromal cells can contribute to the increased levels of MMPs in the tumor microenvironment, but in many cases the stromal cells, such as fibroblasts and macrophages, appear to be the primary source of MMPs (Madsen and Bugge 2015).

The MMP-mediated cleavage of collagen is an extracellular event that has been the subject of numerous studies over the last 50 years, but interestingly an intracellular collagen degradation mechanism, which appears to be centrally engaged in collagen degradation as well, has received much less attention. This pathway involves receptor-mediated internalization of collagen for lysosomal degradation and cooperates with extracellular proteolysis for the complete degradation of insoluble collagen fibers. The rest of this book chapter will focus on this important part of the collagen degradation process.

2.3 Intracellular Collagen Degradation

The possibility that internalization of collagen for lysosomal degradation is a physiologically relevant collagenolytic pathway was suggested more than five decades ago based on electron microscopy evaluation of various tissues (Cullen 1972; Scherft and Heersche 1975; Garant 1976; Beertsen and Everts 1977). In these studies it was observed that in several locations and conditions, cells could display intracellular collagen-containing vacuoles. The intracellular collagen was in the form of crossbanded collagen fibrils strongly suggesting that it had been internalized from the extracellular space and did not represent newly synthesized collagen. These early electron microscopy-based studies on collagen internalization have been reviewed excellently (Everts et al. 1996).

Using ex vivo assays, the ability of cells to internalize collagen has been demonstrated, and these types of studies opened up the possibility to investigate the uptake mechanism in more detail. Upon internalization, collagen is routed to the lysosomes where especially cysteine proteases such as cathepsin B and cathepsin L complete the degradation process (Everts et al. 1985; van Noorden and Everts 1991; Creemers et al. 1998). Obvious candidate cell surface receptors for collagen binding were β1-integrins, which are central collagen receptors that support adhesion and migration on collagen surfaces (Mettouchi and Meneguzzi 2006; Leitinger and Hohenester 2007). β1-integrins are not classical endocytosis receptors, but in several papers they have been described to have an impact on the cellular internalization of collagen-coated fluorescent polystyrene beads mimicking the uptake of large collagen particles (Lee et al. 1996; Segal et al. 2001). More recently, many other studies on cellular collagen internalization have, however, indicated that a

different family of collagen receptors is even more critical for efficient collagen internalization.

2.3.1 The Collagen Internalization Receptor uPARAP/Endo180

About 25 years ago, two independent research groups identified a cell surface receptor, which later turned out to be a novel collagen receptor (Isacke et al. 1990; Behrendt et al. 1993). This receptor was identified as a surface antigen on fibroblasts (Isacke et al. 1990) and as a receptor that could in certain cell types be enzymatically crosslinked to the urokinase receptor (uPAR) (Behrendt et al. 1993). Later on, protein purification, peptide sequencing, and cloning of the full-length cDNA encoding the proteins revealed that the two receptors were indeed the same protein (Behrendt et al. 2000; Sheikh et al. 2000). The receptor was named Endo180 or uPAR-associated protein (uPARAP) (alternative names are CD280 and *MRC2*) and was found to be an endocytic receptor belonging to the mannose family, which consists of four members: the mannose receptor (MR), uPARAP/Endo180, PLA2R, and DEC-205. All four receptors are constitutively active endocytosis receptors that can internalize their ligands and deliver them for lysosomal degradation whereas the receptors recycle back to the cell surface (East and Isacke 2002). The four receptors share a very similar protein domain composition that includes a fibronectin type II (FN-II) domain in the N-terminal part of the protein (Fig. 2.1a). FN-II domains are found in fibronectin and in MMP-2 and MMP-9, and here they mediate the binding to collagen type I (Bányai and Patthy 1991; Bányai et al. 1994). In line with this function of FN-II domains, solubilized collagen can be very efficiently internalized by cultured fibroblasts in a completely uPARAP/Endo180-dependent manner (Fig. 2.1b–c) (Engelholm et al. 2003; East et al. 2003; Kjøller et al. 2004; Madsen et al. 2011). So far collagens of type I, II, IV, V, and VI and gelatin have been tested and found to be ligands for uPARAP/Endo180 (Engelholm et al. 2003; East et al. 2003; Wienke et al. 2003), but no binding to other non-collagenous proteins including fibronectin, laminin, and vitronectin has been observed. It should, however, be noted that it has been demonstrated that fibronectin, when incorporated into a collagen matrix, can be internalized in a uPARAP/Endo180-dependent manner (Shi et al. 2010), probably reflecting the co-internalization of collagen and fibronectin. The collagen-binding capacity of uPARAP/Endo180 is contained within the three N-terminal domains, which include the FN-II domain (Wienke et al. 2003; Jürgensen et al. 2011; Jürgensen et al. 2014). In addition, the fourth protein domain, which is a calcium-dependent lectin-like (CTLD) domain, contributes to the binding to glycosylated collagen forms (Jürgensen et al. 2011).

Using single particle electron microscopy, a low-resolution three-dimensional structure of uPARAP/Endo180 has been solved (Rivera-Calzada et al. 2003; Boskovic et al. 2006). At neutral pH, the four N-terminal domains adopt a bent

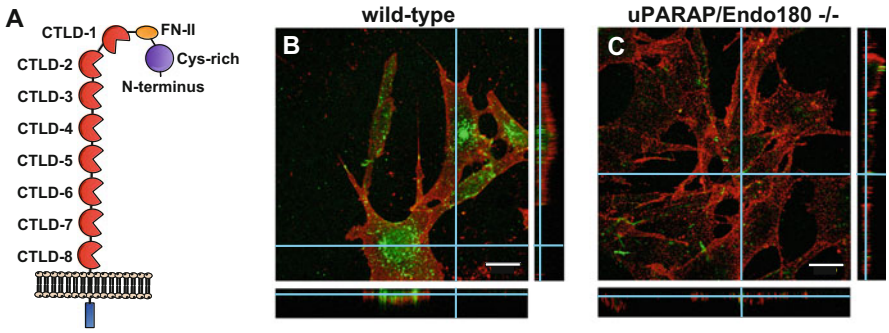

Fig. 2.1 uPARAP/Endo180 and the mannose receptor (MR) are endocytic collagen receptors. (**a**) Schematic representation of the domain composition of uPARAP/Endo180 and MR. uPARAP/Endo180 and MR share an identical domain composition staring with an N-terminal cysteine-rich domain (Cys-rich), followed by a fibronectin type II domain (FN-II), 8 C-type lectin-like domains (CTLDs), a transmembrane-spanning region, and a small cytoplasmic domain. (**b** and **c**) Fibroblasts internalize collagen in a uPARAP/Endo180-dependent manner. Cellular internalization of fluorescent collagen by wild-type (**b**) and uPARAP/Endo180-deficient fibroblasts (**c**) analyzed using confocal microscopy. Cells were incubated with Oregon Green-collagen IV (*green*) to allow endocytosis, followed by cell surface staining (*red*). The intracellular punctuate pattern of collagen indicates localization in endosomal/lysosomal compartments. Scale bar, 20 μm. (**b** and **c**) were adapted from Kjøller et al. (2004) with permission from Elsevier

conformation, where the cysteine-rich (Cys-rich) domain contacts CTLD-2, but at pH 5.4 the receptor acquires a more extended conformation. This conformational change might relate to the ligand release mechanism of the receptor in the slightly acidic endosomes. Recently a high-resolution crystal structure of a truncated version of uPARAP/Endo180, consisting of the four N-terminal domains, has been solved (Paracuellos et al. 2015). This part of uPARAP/Endo180 forms a more open L-shaped structure that is unaffected by changes in pH, suggesting that the previously observed bent confirmation might instead involve an interaction between the N-terminal Cys-rich domain and CTLD-3 (Paracuellos et al. 2015).

In cellular assays in which fibroblasts are presented with solubilized forms of collagen, efficient and completely uPARAP/Endo180-dependent collagen internalization occurs. Furthermore, the uptake of solubilized collagen is independent of β1-integrin activity, which is in contrast to the reports on cellular uptake of collagen-coated beads (Madsen et al. 2011). The observed differences most likely reflect the different assay systems used. When fibroblasts are presented with an insoluble collagen matrix resembling native collagen fibers, uptake of collagen material occurs in a process that appears to be dependent on both uPARAP/Endo180 and β1-integrins (Shi et al. 2010). Strikingly, however, it has been demonstrated in another study that the conditioned media from uPARAP/Endo180-deficient fibroblasts cultured on a collagen matrix contain collagen fragments that correspond to the one quarter and three quarter fragments generated by MMP-mediated cleavage (Fig. 2.2a–b) (Madsen et al. 2007). In contrast, littermate wild-type fibroblasts have the ability to internalize and degrade the released

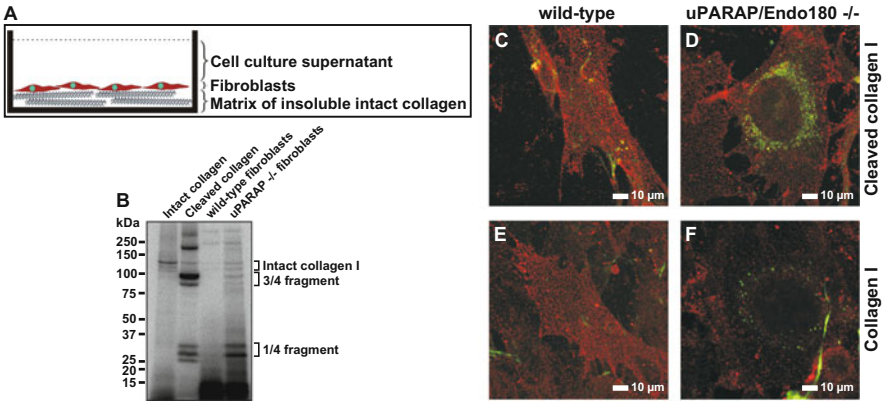

Fig. 2.2 Fibroblasts internalize MMP-generated collagen fragments in a uPARAP/Endo180-dependent manner. (**a** and **b**) uPARAP/Endo180 is indispensable for complete degradation of insoluble collagen fibers by fibroblasts. (**a**) Schematic overview of the experimental setup used for investigating the complete process of cellular collagen degradation. Fibroblasts were cultured for 5 days on top of an insoluble collagen matrix with a tracer of radiolabeled collagen included after which the cell culture supernatant was analyzed for the presence of collagen fragments. (**b**) SDS-PAGE and phosphorimager analysis shows that collagen fragments accumulate in the conditioned media of uPARAP/Endo180-deficient fibroblasts (lane 4). These fragments are cleared from the conditioned media by wild-type fibroblasts (lane 3). Intact collagen (lane 1) and MMP-13-generated collagen fragments (lane 2) are included for comparison. (**e**–**f**) MMP-generated collagen fragments are internalized more efficiently than intact collagen. Cellular internalization of MMP-13-cleaved (**c** and **d**) and intact (**e** and **f**) fluorescent collagen type I (*green*) by wild-type (**c** and **e**) or uPARAP/Endo180-deficient fibroblasts (**d** and **f**). A was adapted from Madsen et al. (2011), and (**b**–**f**) were adapted from Madsen et al. (2007)

collagen fragment, thereby leaving no large collagen fragments in the conditioned media. This strongly suggests that fibroblasts degrade collagen in a sequential process initiated by MMP-mediated cleavage of insoluble collagen fibers, followed by uPARAP/Endo180-mediated uptake of the released one quarter and three quarter collagen fragments. In accordance with these findings, biochemical analysis of the protein interaction between uPARAP/Endo180 and collagen revealed that collagen upon denaturation binds with an increased association rate to uPARAP/Endo180 (Leitinger and Hohenester 2007; Madsen et al. 2007; Jürgensen et al. 2011; Paracuellos et al. 2015). The one quarter and three quarter collagen fragments, which shift to a partially denatured state at 37 °C (Stark and Kühn 1968; Danielsen 1987), similarly show a more rapid binding to uPARAP/Endo180 compared to intact full-length collagen (Madsen et al. 2007). Consistently, MMP-generated collagen fragments are also internalized more efficiently than intact collagen (Fig. 2.2c–f).

The initial fragmentation of the insoluble collagen matrix is mainly mediated by MT1-MMP (Lee et al. 1996; Holmbeck et al. 1999; Ingvarsen et al. 2013), and this process is very likely facilitated by β1-integrin-mediated cellular interactions with the matrix (Segal et al. 2001; Hynes 2002; Shi et al. 2010). In addition, the

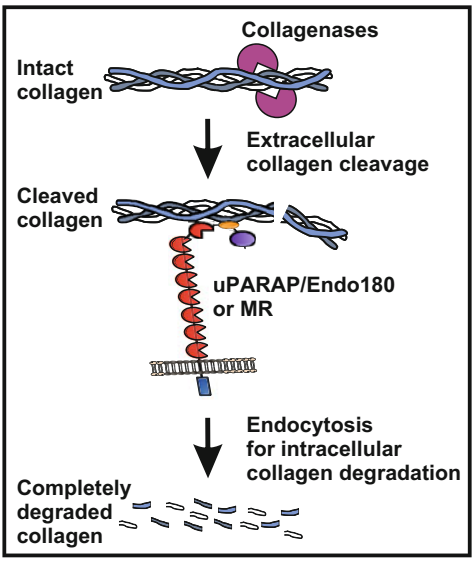

Fig. 2.3 Collagen is degraded in a sequential process that requires receptor-mediated collagen internalization. Schematic representation of the collagen degradation process. The degradation process is initiated by proteolytic attack of insoluble collagen fibers to release defined soluble collagen fragments. This extracellular cleavage reaction is primarily mediated by the collagenolytic MMPs, such as MT1-MMP. The released collagen fragments bind to MR or uPARAP/Endo180 on the surface of M2 macrophages or fibroblast-like cells, respectively. The collagen fragments are internalized and routed to the lysosomes for complete degradation. Cysteine cathepsins are essential for the lysosomal degradation of collagen

membrane-anchored serine protease FAP can facilitate collagen internalization by participating in the further fragmentation of one quarter and three quarter collagen fragments (Fan et al. 2015). The internalized collagen fragments are finally routed to the lysosomes for complete degradation through the action of cysteine cathepsins (Kjøller et al. 2004; Mohamed and Sloane 2006; Madsen et al. 2007).

Altogether it is suggested that fibroblast-mediated collagen degradation occurs in a sequential process initiated by MMP-mediated cleavage of large insoluble collagen fibers followed by uPARAP/Endo180-mediated uptake of the released collagen fragments for lysosomal degradation (Fig. 2.3).

2.3.2 The Collagen Internalization Receptor MR

uPARAP/Endo180 was initially identified as a novel fibroblast antigen, and in culture essentially all mesenchymal cells express uPARAP/Endo180. In addition to fibroblasts, this includes osteoblasts, chondrocytes, hepatic stellate cells, pancreatic stellate cells, glioma cells, and various sarcoma cell lines (Mousavi et al. 2005; Wagenaar-Miller et al. 2007; Mousavi et al. 2009; Huijbers et al. 2010; Madsen

et al. 2011; Takahashi et al. 2011; Madsen et al. 2012; Ikenaga et al. 2012). uPARAP/Endo180 shows the highest degree of homology with the mannose receptor (MR, alternative names are CD206 and *MRC1*), which is a receptor that is primarily expressed by subsets of macrophages and by liver sinusoidal endothelial cells. MR has mainly been investigated as a receptor for certain pathogens and a potential key receptor involved in innate immunity. Based on the homology with uPARAP/Endo180, it was hypothesized that collagen could be a novel ligand for MR, and it has indeed been demonstrated that MR can bind and internalize collagens with efficiency and binding specificity very similar to that of uPARAP/Endo180 (Martinez-Pomares et al. 2006; Malovic et al. 2007; Jürgensen et al. 2011, 2014; Madsen et al. 2011). The collagen-binding capacity of MR is localized to the FN-II domain containing N-terminus (Napper et al. 2006; Jürgensen et al. 2014), and it has been suggested that multimerization of the receptor is required for efficient binding of collagen on the cell surface (Martinez-Pomares et al. 2006). In a direct comparison of the four receptors from this family, it has been shown that only uPARAP/Endo180 and MR possess the ability to bind and internalize collagen (Jürgensen et al. 2014). Although PLA2R and DEC-205 share a similar domain composition, including the presence of an FN-II domain, these two receptors are completely unable to internalize collagen (Jürgensen et al. 2014). The ability to bind collagen is mediated by a protruding ten-residue collagen-binding loop of the FN-II domain as well as adjacent protein domains, which probably affect either the multimerization of the receptor or the formation of a binding-active conformation (Jürgensen et al. 2014).

Using single particle electron microscopy, the N-terminal part of MR has been demonstrated to adopt a bent three-dimensional structure (Boskovic et al. 2006). Overall, the structure appears very similar to the structure of uPARAP/Endo180 (Rivera-Calzada et al. 2003; Boskovic et al. 2006) although the globular N-terminal part of MR involves two more CTLDs than observed for uPARAP/Endo180. Similar to uPARAP/Endo180, MR also adopts a different conformation at a lower pH corresponding to the endosomal environment (Boskovic et al. 2006).

2.4 Intracellular Collagen Degradation In Vivo

Most mesenchymal cells express uPARAP/Endo180 in vitro, and in vivo the expression of uPARAP/Endo180 is also restricted to certain mesenchymal cells. By Northern blotting and Western blotting of various tissues from mice, it has been shown that most tissues express uPARAP/Endo180, with especially high expression levels observed in the heart, lung, uterus, and bones (Wu et al. 1996; Madsen et al. 2013a). Very low expression levels are detected in the liver and brain. Histological analyses have demonstrated that uPARAP/Endo180 is expressed by mesenchymal cells of the developing and adult murine lung (Smith et al. 2008; Bundesmann et al. 2012) and in osteoblasts and chondrocytes of developing and adult bone (Engelholm et al. 2001; Howard et al. 2004; Wagenaar-Miller et al.

2007; Madsen et al. 2013a; Abdelgawad et al. 2014). In the skin, macrophages as well as fibroblasts, pericytes, and endothelial cells have been reported to express uPARAP/Endo180 (Sheikh et al. 2000; Honardoust et al. 2006). In human liver fibrosis and in mouse models of lung and liver fibrosis, uPARAP/Endo180 is upregulated and expressed by myofibroblasts (Madsen et al. 2012; Bundesmann et al. 2012). In mouse models of kidney fibrosis, uPARAP/Endo180 is expressed by myofibroblasts and a subset of macrophages (López-Guisa et al. 2012). In general, uPARAP/Endo180 is upregulated in conditions known to involve increased tissue remodeling, including bone development (Engelholm et al. 2001; Howard et al. 2004; Wagenaar-Miller et al. 2007), skin wound healing (Rohani et al. 2014), tissue fibrosis (Madsen et al. 2012; Bundesmann et al. 2012; López-Guisa et al. 2012), and in connection to cancer as described in detail later in this book chapter. In bone development, the importance of uPARAP/Endo180 became particularly clear when inactivating mutations in the *MRC2* gene coding for uPARAP/Endo180 were identified as the underlying cause of a severe hereditary skeletal disorder in cattle known as crooked tail syndrome (CTS) (Fasquelle et al. 2009; Sartelet et al. 2012). In mice, uPARAP/Endo180 deficiency only results in a relatively mild skeletal phenotype (Wagenaar-Miller et al. 2007; Madsen et al. 2013a), but when combined with deficiency of the important extracellular collagenase MT1-MMP, severely impaired bone development is observed, and the mice only have a life span of up to 3 weeks (Wagenaar-Miller et al. 2007). So far, no human inactivating mutations of uPARAP/Endo180 have been reported although a single nucleotide polymorphism (SNP) in a regulatory region of the *MRC2* gene has been found to associate with degenerative bony changes of the temporomandibular joint (Yamaguchi et al. 2014), and a SNP in exon 30 of *MRC2* associates with the risk of recurrent disease for head and neck squamous carcinoma patients (Wu et al. 2009).

MR was first identified on Kupffer cells (Schlesinger et al. 1978) and later was also found to be expressed by many other types of tissue macrophages (Takahashi et al. 1998) and by subsets of dendritic cells (Linehan 2005; Burgdorf et al. 2006). In addition to macrophages, MR is expressed at high levels by sinusoidal endothelial cells in the liver and in lymph nodes (Takahashi et al. 1998; Malovic et al. 2007). Early studies of the function of the MR primarily focused on its potential role in the innate immune system as a receptor for certain pathogens due to the carbohydrate-binding properties of the receptor. However, subsequent littermate-controlled studies of MR knockout mice have failed to clearly demonstrate any significant changes in susceptibility to these pathogens (Lee et al. 2003; Swain et al. 2003).

Instead, the first study reporting the generation of MR knockout mice revealed increased serum levels of several glycoproteins including seven lysosomal hydrolases and the carboxy-terminal propeptide domains of collagen types I and III in MR-deficient mice (Lee et al. 2002). MR-deficient mice also have a significantly reduced ability to clear denatured collagen from the circulation (Malovic et al. 2007). In subsets of dendritic cells, MR probably contributes to the cross presentation of certain glycosylated antigens (Burgdorf et al. 2006), and interestingly the

MR-mediated uptake of these antigens can be inhibited by collagen fragments (Burgdorf et al. 2010).

Still, the study of MR as a collagen internalization receptor involved in ECM turnover is in its infancy, and future in vivo studies addressing the role of MR in tissue remodeling processes are therefore a high priority. However, as described in detail in the section below, a recent study strongly suggests that MR-mediated collagen internalization could be an important function of M2 macrophages in the dermis and in tumors of mice (Madsen and Bugge 2013; Madsen et al. 2013b).

2.4.1 Imaging Collagen Internalization In Vivo

As outlined earlier in this book chapter, in vitro studies have clearly shown that cells can internalize collagen for lysosomal degradation in a uPARAP/Endo180- or MR-dependent manner. Furthermore, in vivo studies have revealed phenotypes of uPARAP/Endo180-deficient animals consistent with a role of this receptor in collagen remodeling processes. However, many aspects related to the mechanism of collagen internalization in vivo are still uncovered, although some answers have been provided by a recent study utilizing a novel confocal microscopy assay to analyze the cellular uptake of collagen in the skin of mice (Madsen et al. 2013b). In this study fluorescent collagen was introduced into the connective tissue of the skin of mice where it immediately polymerized to form insoluble collagen fibers indistinguishable from endogenous collagen fibers. The turnover of these collagen fibers could be visualized using two-photon or confocal microscopy analysis of the intact tissue. The study revealed that fluorescent collagen or collagen degradation fragments are efficiently internalized by various cells in the dermis (Fig. 2.4a). By combining the assay with transgenic mice expressing cell lineage-specific fluorescent reporter proteins or with whole-mount immunostaining, it was demonstrated that several cell types are capable of internalizing collagen, including fibroblasts and Cx3Cr1-positive macrophages, but that M2-like macrophages are the dominant collagen-internalizing cell population (Fig. 2.4b). These cells are positive for the pan-macrophage marker F4/80 and for the M2-macrophage markers Fizz1 and MR. Collagen internalization by these M2-like macrophages is mediated by MR as demonstrated by the lack of collagen internalization in MR-deficient mice (Madsen et al. 2013b). M2-polarized macrophages are cells proposed to play an important role in tissue remodeling and repair processes, but this has mainly been ascribed to their ability to stimulate ECM synthesis (Ingman et al. 2006) and to dampen inflammatory reactions (Gordon and Martinez 2010; Vasievich and Huang 2011). Internalization of collagen, and potentially other ECM proteins, is likely to be another important way by which these cells promote tissue remodeling. Since tumor-associated macrophages (TAMs) often acquire an M2-polarized phenotype (Biswas et al. 2013), it has been suggested that TAMs play an important role for the collagen degradation that occurs in connection to solid tumor growth and invasion (Madsen and Bugge 2013). Recently we have indeed been able to demonstrate,

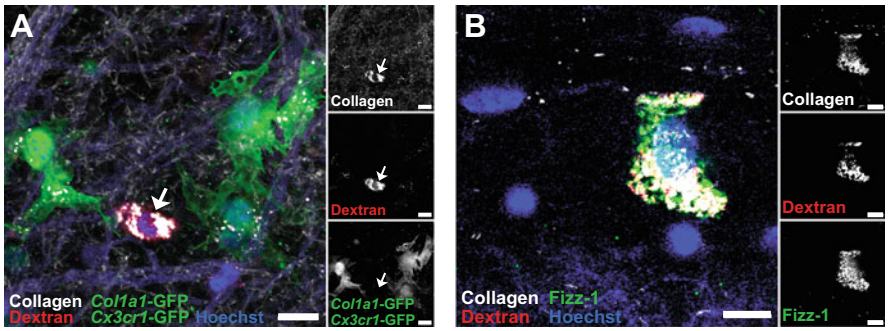

Fig. 2.4 M2 macrophages of the dermis internalize high levels of collagen. (**a**) Image of the dermis from collagen-injected *Cx3cr1*-GFP;*Col1a1*-GFP double transgenic mice. Fluorescent collagen (*white*) and dextran (*red*) were introduced into the dermis of transgenic reporter mice expressing GFP in *Cx3cr1*-positive monocytes and *Col1a1*-positive fibroblast (*green*). 24 h later the dermis was excised and the fresh tissue was imaged using confocal microscopy. Several cell population display intracellular collagen-containing vesicles, but certain cells that were not monocytes or fibroblasts internalized very high levels of collagen and dextran (*white arrow*). Scale bar, 20 μm. (**b**) High-level collagen-internalizing cells of the dermis express the M2-macrophage marker Fizz-1. Fluorescent collagen (*white*) and dextran (*red*) were introduced into the dermis of mice, and 20 h later the dermis was excised and whole mount stained for Fizz-1 (*green*). Adapted from Madsen et al. (2013b)

using a similar confocal microscopy-based assay, that in solid tumors of mice, M2-polarized TAMs are the dominant collagen internalizing cells (Madsen et al. unpublished). These collagen-internalizing TAMs are very abundant in the tumors and internalize collagen in an MR-dependent manner (Madsen et al. unpublished).

2.4.2 Intracellular Collagen Degradation in Cancer

In most tissues, resting or quiescent fibroblasts express no or very low levels of uPARAP/Endo180, whereas activated myofibroblasts in connection to cancer are uPARAP/Endo180 positive (Fig. 2.5a–b) (Schnack Nielsen et al. 2002; Curino et al. 2005; Sulek et al. 2007; Kogianni et al. 2009; Melander et al. 2015). Such uPARAP/Endo180-positive cancer-associated fibroblasts (CAFs) have been demonstrated in all studied carcinomas, including breast cancer, prostate cancer, and head and neck squamous cell carcinoma. In osteosarcomas and gliomas, the cancer cells themselves often express uPARAP/Endo180 (Huijbers et al. 2010; Takahashi et al. 2011; Engelholm et al. 2016), and in a small subset of breast tumors with a basal-like subtype, the cancer cells are also uPARAP/Endo180 positive (Wienke et al. 2007). In prostate cancer specimens, an upregulation of uPARAP/Endo180 has been demonstrated, with expression suggested to be both on cancer cells and stromal cells (Kogianni et al. 2009). In addition to these descriptive studies, a few studies have demonstrated the direct functional implications of uPARAP/Endo180 for

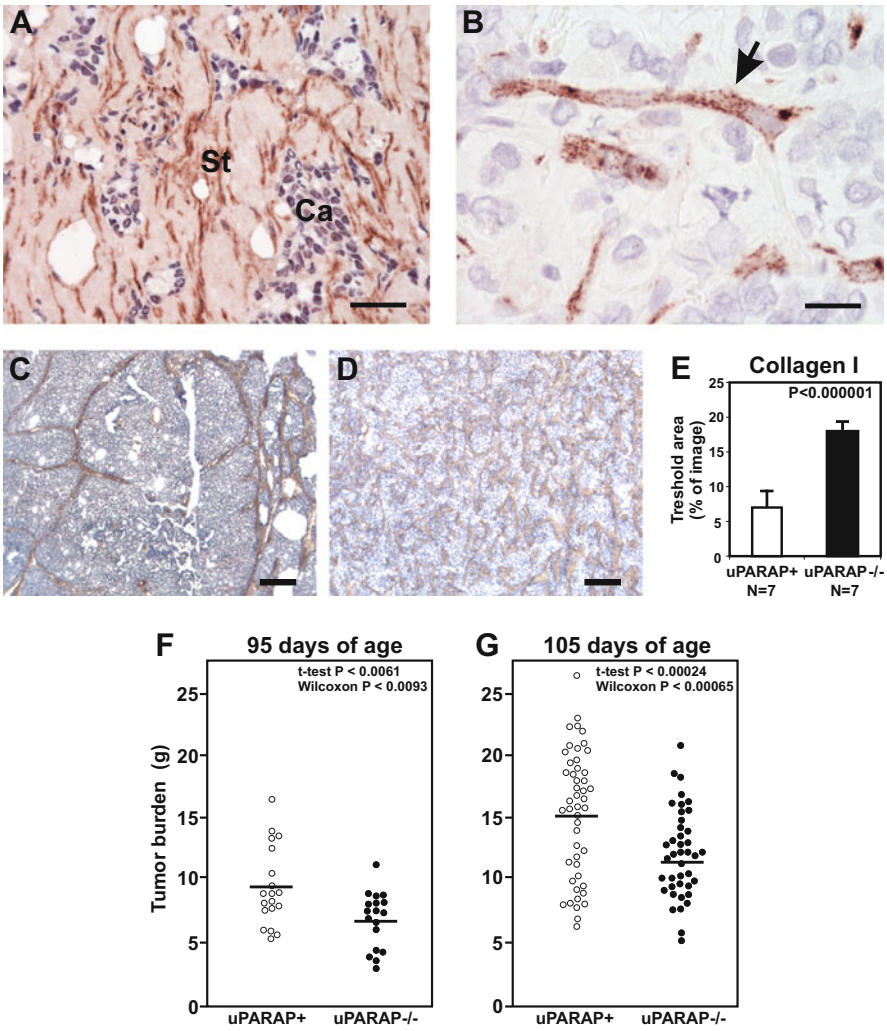

Fig. 2.5 uPARAP/Endo180-mediated collagen remodeling promotes tumor growth. uPARAP/Endo180 is upregulated in human breast cancer (**a** and **b**). (**a**) Immunohistochemical analysis of uPARAP/Endo180 in invasive ductal breast carcinoma specimens demonstrates upregulation of uPARAP/Endo180 (*brown*) with expression restricted to the stromal cells of the tumor (St). uPARAP/Endo180-positive fibroblast-like cells show a punctuated staining pattern (*arrow*) indicative of a localization in intracellular vesicles (**b**). Intratumoral collagen accumulates in uPARAP/Endo180-deficient mice in a genetic mouse model of breast cancer. Transgenic PymT mice that are predisposed to develop breast cancer were crossed with uPARAP/Endo180-deficient mice. (**c** and **d**) Immunohistochemical analysis of collagen type I reveals large accumulations of collagen type I (*brown*) in tumors of uPARAP/Endo180-deficient mice (**d**) compared to tumors of littermate uPARAP/Endo180-expressing mice (**c**). Scale bar, 500 μm. (**e**) Histomorphometric quantification of collagen type I in the tumors of uPARAP/Endo180-expressing mice (*white bar*) and littermate uPARAP/Endo180-deficient mice (*black bar*). (**f** and **g**) uPARAP/Endo180 promotes breast tumor growth. Scatter plots illustrate the cumulative tumor burden of uPARAP/Endo180-expressing mice (*open circles*) and uPARAP/Endo180-deficient mice (*filled circles*) at 95 (**f**) and 105 (**g**) days of age. (**a**) and (**b**) were adapted from Schnack Nielsen et al. (2002) with permission from

tumor growth. First of all, a study by Curino et al. has investigated the effects of uPARAP/Endo180 deficiency in a genetic mouse model of breast cancer. In this model, the tumors of uPARAP/Endo180 knockout mice are smaller than the tumors of littermate uPARAP/Endo180-expressing mice, and strikingly the uPARAP/Endo180-deficient mice display highly increased levels of intratumoral collagen (Fig. 2.5c–g) (Curino et al. 2005). When collagen internalization by ex vivo explants from these tumors was assayed, it was observed that fibroblasts from uPARAP/Endo180-deficient tumors lack the ability to internalize collagen. In another study it has been demonstrated that MCF-7 breast cancer cells transfected with uPARAP/Endo180 have a growth advantage over cells transfected with an internalization-deficient mutant when inoculated into immunocompromised mice (Wienke et al. 2007). Using antibody-mediated neutralization of uPARAP/Endo180, it has recently been demonstrated that uPARAP/Endo180 expressed by sarcoma cells in mice contributes to the severe bone degradation that accompanies the tumor progression (Engelholm et al. 2016). Altogether these studies demonstrate that uPARAP/Endo180 affects ECM remodeling associated with tumor progression and influences tumor growth and associated skeletal morbidities.

Historically, MR has been studied as a receptor involved in the innate immune response, and its importance in vivo as a collagen internalization receptor with a potential role in tumor growth and invasion still remains to be addressed. In recent years, MR has been given a lot of attention as a marker for M2-polarized macrophages (Gordon and Martinez 2010). M1 macrophages, also known as classically activated macrophages, develop when exposed to the pro-inflammatory cytokine interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNFα), whereas M2 macrophages, also known as alternatively activated macrophages, develop in response to interleukin-4 (IL-4) and IL-13 (Gordon and Martinez 2010; Wang and Joyce 2010). M2 macrophages are anti-inflammatory macrophages that are often engaged in tissue remodeling processes such as wound healing (Martinez et al. 2009). In connection to cancer, it has been proposed that M2 macrophages have pro-tumorigenic functions through their ability to promote tumor-associated angiogenesis and their ability to stimulate cancer cell migration and invasion (Noy and Pollard 2014). M2 macrophages also have immunosuppressive functions and can contribute to a tumor microenvironment, which helps the tumor cells evade a T-cell response directed against them (Vasievich and Huang 2011; Noy and Pollard 2014). At this point, the role of MR expressed by M2 macrophages for tumor growth and invasion still remains to be directly investigated. However, the recent demonstration of MR as a critical receptor for macrophage-mediated collagen uptake in the skin and in tumors of mice (Madsen et al. 2013b; Madsen et al. unpublished) renders it highly probable that MR could play an important role for tumor-associated collagen remodeling and thereby critically affect tumor growth

Fig. 2.5 (continued) John Wiley and Sons, and C-G were adapted from Curino et al. (2005) with permission from Rockefeller University Press

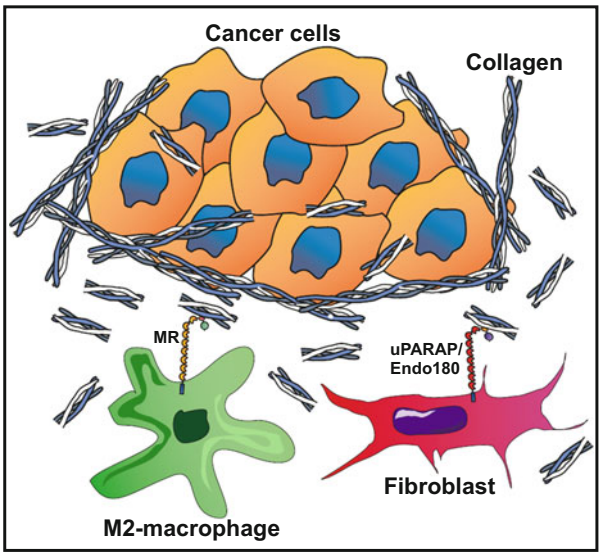

Fig. 2.6 Tumor-infiltrating fibroblasts and M2 macrophages promote collagen degradation. Simplified schematic representation of a tumor consisting of cancer cells, collagen, fibroblasts, and M2 macrophages. In connection to invasive tumor growth, fibroblasts and M2 macrophage contribute to the tumor-associated ECM remodeling. Fibroblasts and M2 macrophages can internalize collagen fragments for lysosomal degradation through the action of uPARAP/Endo180 and MR, respectively

(Fig. 2.6). Future studies will reveal the importance of MR-mediated collagen degradation in cancer and in connection to other pathological conditions and indicate whether therapeutic targeting of MR could form the basis of novel cancer treatments.

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Chapter 3
Lumican, a Small Leucine-Rich Proteoglycan, and Its Biological Function in Tumor Progression

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Abstract Lumican is a member of the small leucine-rich proteoglycan (SLRP) family that was originally discovered in the chick cornea and is found in many other tissues throughout the human body. The SLRP family includes decorin, lumican, biglycan, and fibromodulin and constitutes an abundant component of the extracellular matrix (ECM). Lumican plays a significant role in the ECM as an organizer of collagen, although recent studies demonstrate that lumican also modulates numerous cellular functions including proliferation, migration, and differentiation. The contribution of lumican to cancer progression has been noted in several cancers including breast, colorectal, and pancreatic; however, its precise biologic function is still being uncovered. In cancer, lumican appears to play a context-specific role, where high levels of lumican are associated with a poor prognosis in some cancers and a better prognosis in others. This chapter focuses on the function of lumican in cell biology and the ECM of solid tumors and is aimed at providing insights into molecular mechanisms surrounding lumican and tumor biology.

3.1 Structure, Function, and Regulation of Lumican

Lumican, also known as LDC or SLRR2D, is located at chromosome 12q21.33 (Chakravarti et al. 1995). It is a member of the small leucine-rich proteoglycan (SLRP) family that also includes decorin, biglycan, fibromodulin, keratocan, epiphycan, and osteoglycin. Lumican was originally characterized as one of the major keratan sulfate (KS)-containing proteoglycans and was initially purified by DEAE chromatography from the chick cornea; its distribution is now known to include interstitial collagenous matrices throughout the body (Blochberger et al. 1992a, b). Lumican contains an 18-amino acid signal peptide that facilitates secretion, followed by an N-terminal domain containing four cysteines, a ~40 kDa core

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protein with a central domain containing 6–10 characteristic leucine repeats (LLRs; Chakravarti et al. 1995), and a C-terminal domain with two cysteines and two LRRs. Lumican also contains four potential N-linked glycosylation sites distributed across the protein. When lumican assumes an arch-shaped tertiary structure (Kajava 1998), these glycosylation sites are presented on the convex surface, while the concave surface binds collagen and facilitates spacing between fibers (Kalamajski and Oldberg 2009; Weber et al. 1996).

Lumican was originally characterized for its role in collagen fibrillogenesis and structural organization (Chakravarti et al. 1998). Deficiencies in lumican result in abnormal collagen fibrillogenesis, which affects connective tissue structure and function (Nikitovic et al. 2008b). In the cornea, lumican not only organizes collagen (Chakravarti et al. 1998) but also influences corneal epithelial wound healing (Liu and Kao 2012). Healing of corneal epithelium of $Lum^{-/-}$ mice was significantly delayed compared to that of wild-type mice, and lumican was ectopically and transiently expressed in the corneal epithelium during the early stages of wound healing. In addition to controlling collagen fibril assembly, lumican participates in the regulation of key biological events including cell proliferation (Ishiwata et al. 2004; Pietraszek et al. 2013), migration (Nikitovic et al. 2008a; Lee et al. 2009; Fullwood et al. 1996), and adhesion (D'Onofrio et al. 2008; Brezillon et al. 2009; Cole and Mccabe 1991; Liu and Kao 2012). However, the opposite effects were noted in human embryonic kidney 293 (HEK293) cells, where cells stably expressing and secreting lumican showed decreased adhesion and growth compared to mock HEK293 cells, while migration and invasion were seemingly unaffected (Ishiwata et al. 2010). Lumican has also been implicated in the inhibition of matrix metalloproteases (MMPs). Specifically, the glycosylated form of lumican decreased MMP-14 activity in B16F1 melanoma cells. Lumican may protect collagen against MMP-14 proteolysis, thus influencing cell-matrix interaction in tumor progression. (Pietraszek et al. 2014; Niewiarowska et al. 2011; Pietraszek et al. 2013) and has also been implicated as an inhibitor of angiogenesis (Niewiarowska et al. 2011; Nikitovic et al. 2014; Sharma et al. 2013).

The complexity and diversity of its proteoglycan structure suggest that lumican may influence cell function through several mechanisms. Lumican can present itself in a variety of forms depending on glycosylation. A highly substituted form of lumican has been identified within aortic smooth muscle cells in rats (Qin et al. 2001), with serum analysis revealing proteoglycan, glycoprotein, and core protein forms. Recently, the importance in lumican glycosylation in aortic valve stenosis (AS) has begun to be studied (Suzuki et al. 2016). Insufficient glycosylation of lumican was associated with thickened and calcified regions of AS valves, potentially due to the impairment of collagen fibrils and induction of inflammation. In lung tissues, lumican was found in a large number of different glycosylation states. These studies demonstrated, however, that the glycosylation pattern of secreted lumican is much more uniform than intracellular forms, suggesting a requirement for a more uniform protein type when lumican is in the extracellular space. Lumican forms can also depend upon age. Within the ECM of articular cartilage (Grover et al. 1995), the highly substituted keratan sulfate proteoglycan form of

lumican is much more prevalent in juvenile tissues, whereas the keratan sulfate-lacking glycoprotein is correlated with adults. Interestingly, there is a higher abundance of lumican in adults, despite having the less substituted form. These differences suggest that lumican forms and function differ depending upon age and anatomic location.

3.2 Lumican Implications in Cancer

During the many steps of tumor metastasis, cancer cells must interact with their microenvironment to grow, invade locally, intravasate into blood and lymphatic vessels, migrate, and grow again at anatomically distant sites (Hanahan and Weinberg 2011). Throughout these events, cancer cells interact with the components of the extracellular matrix (ECM), growth factors, and cytokines associated with the ECM, as well as surrounding stromal cells (endothelial cells, fibroblasts, macrophages, mast cells, neutrophils, pericytes, and adipocytes; Bhowmick et al. 2004; Lu et al. 2012). Modifications of ECM components during tumor progression have been extensively reported, and the role of proteoglycans in particular has been emphasized recently. Early studies have evaluated the effect of lumican on the proliferation and metastasis of several cancers (Naito 2005; Fullwood et al. 1996; Wight et al. 1992), but further studies into the biological mechanism of its effect on cancer are still needed. The presence of lumican has been observed in breast, colorectal, lung, melanoma, prostate, and pancreas cancers (Leygue et al. 1998; Lu et al. 2002; Matsuda et al. 2008; Ping Lu et al. 2002; Pietraszek et al. 2013; Li et al. 2016; Yang et al. 2013; Suhovskih et al. 2013; Coulson-Thomas et al. 2013; Seya et al. 2006), among others. However, a consensus on whether lumican has a positive or negative impact on tumor dynamics has not been reached.

3.2.1 Breast Cancer

Lumican in breast cancer has been localized to the tumor stroma and fibroblasts surrounding the lesion, though not in the cancer cells themselves (Leygue et al. 1998). There is little to no expression of lumican in normal breast tissue, providing strong evidence that it plays a role in breast tumor formation. A high expression level of lumican in breast cancer is correlated to high tumor grade, low estrogen receptor levels, and younger patient age. The lumican observed in these tumors presents itself in an unsulfated state. The poorly sulfated form of this protein has been shown to induce macrophage attachment and spreading (Funderburgh et al. 1997), indicating that lumican may participate in macrophage recruitment in these tumors. Further studies are necessary to elucidate the influences and consequences of lumican in breast cancer.

3.2.2 Colorectal Cancer

Lumican in colorectal cancer, on the other hand, is strongly expressed in the cancer cells themselves (Lu et al. 2002). No evidence of lumican was detected in normal epithelial cells, but those within close proximity of a lesion were shown to have weak expression of lumican. This suggests that the cancer cells may influence the surrounding tissues to synthesize lumican in an effort to promote cancer cell growth. As in breast cancer studies, the lumican extracted from these cells was poorly sulfated, strengthening the notion that it contributes to cancer cell proliferation.

3.2.3 Lung Cancer

Lumican in lung cancer has been studied in squamous cell carcinoma (SqCC) and lung adenocarcinoma (ADC), with differing conclusions (Matsuda et al. 2008). Lumican is present in normal lung tissues, specifically peribronchial connective tissues and the bronchial epithelium (Dolhnikoff et al. 1998). However, enhanced expression is detected in stromal tissues and in cancer cells for SqCC and ADC. SqCC showed higher levels in the cancer cells than the stromal tissues, whereas ADC showed higher levels in stromal tissues than cancer cells. In either disease, secreted lumican was found to be variably and abnormally glycosylated, a feature which has been linked to malignant transformation (Kannagi et al. 2004). However, the glycosylation pattern was much more uniform within the cancer cells. This difference suggests different roles for lumican between cancer cells and stromal tissues. A particularly interesting finding was the increased vascular invasion in the presence of lumican in SqCC. While the effect of lumican on angiogenesis has been observed, most literature suggests lumican is an inhibitor of angiogenesis (Albig et al. 2007; Niewiarowska et al. 2011). A possible explanation for this apparent inconsistency is that the majority of lumican in SqCC is secreted by the cancer cells, while the studies that imply angiogenic inhibition focused on epithelial cell expression of the protein (Kannagi et al. 2004). These observations underscore the importance of the context within which lumican is studied. Additionally, these results highlight that lumican exerts its influence on cancer through microenvironmental cues that are still largely unknown (Sharma et al. 2013).

3.2.4 Pancreatic Cancer

More extensive research has been conducted on the role of lumican in pancreatic ductal adenocarcinoma (PDAC). Lumican is expressed in normal pancreas tissues, localized primarily in the alpha cells of islets (Ping Lu et al. 2002). Aberrant

expression of lumican has been observed in stromal cells and cancer cells of PDAC, with differing patient prognoses depending on location (Ishiwata et al. 2007). It was noted that patients with lumican-positive cancer cells had longer survival than those with lumican-negative cancer cells, while patients with lumican-positive stroma tended to survive for a shorter period than those that had stroma devoid of lumican. However, a separate study noted an association between stromal lumican and prolonged survival after surgery (Li et al. 2014). It should be noted that patient tumors studied in these two reports were at very different stages of disease, with the poor outcome identified in later-stage tumors versus the opposite trend identified in earlier-stage tumors. This observation adds tumor stage as an additional consideration to anatomic site when considering lumican in cancer. A study by Yang et al. focused on lumican expression in patients with PDAC and noted exactly this shift in effect and prognosis. Stromal expression of lumican was significantly higher in patients with later stages of disease and correlated with lower expression of Ki-67, vascular endothelial growth factor (VEGF), and mutated p53 (Yang et al. 2013).

Additional functional studies using PDAC cells identified that extracellular lumican stimulates epidermal growth receptor (EGFR) dimerization and internalization, resulting in decreased EGFR kinase activity and attenuation of its downstream activators Akt and HIF-1α. Reduced HIF-1α inhibits glycolytic metabolism and triggers apoptotic cell death (Li et al. 2014). More recently, we further demonstrated that extracellular lumican decreased AMP-activated kinase activity, inhibiting chemotherapy-induced autophagy in in vitro and in vivo PDAC models. Co-treatment of PDAC cells with lumican and gemcitabine increased mitochondrial damage, reactive oxygen species production, and cytochrome c release, indicating that lumican-induced disruption of mitochondrial function may be the mechanism of sensitization to gemcitabine (Li et al. 2016). Our data also identified pancreatic stellate cells (PSCs) as a significant source of extracellular lumican production through quantitative immunohistochemistry analysis of 27 PDAC patient specimens. We demonstrated that the cytokine, transforming growth factor-β (TGF-β), negatively regulates lumican gene transcription within human PSCs through its canonical signaling pathway and binding of SMAD4 to novel SMAD-binding elements identified within the promoter region. Extracellular lumican enhances stellate cell adhesion and mobility in a collagen-rich environment. Pan02 mouse cells have been injected into the lumican$^{-/-}$ pancreas of mice. Histologically, Pan02 cells grew a more moderately differentiated spherical growth pattern (Fig. 3.1a, b) in C57/BL6 wild-type mice, while Pan02 grew a more irregular finger-like or undifferentiated growth pattern in lumican$^{-/-}$ mice and showed local invasiveness (Fig. 3.1c, d; data not published). Figure 3.2 summarizes extracellular lumican regulation and biological functions in PDAC proliferation, apoptosis, adhesion, and migration.

In summary, it is clear that lumican plays an active role in many solid tumors. While its role in cell signaling is being elucidated, understanding how lumican functions as a cell matrix modulator with respect to drug delivery and tumor dynamics is of critical importance.

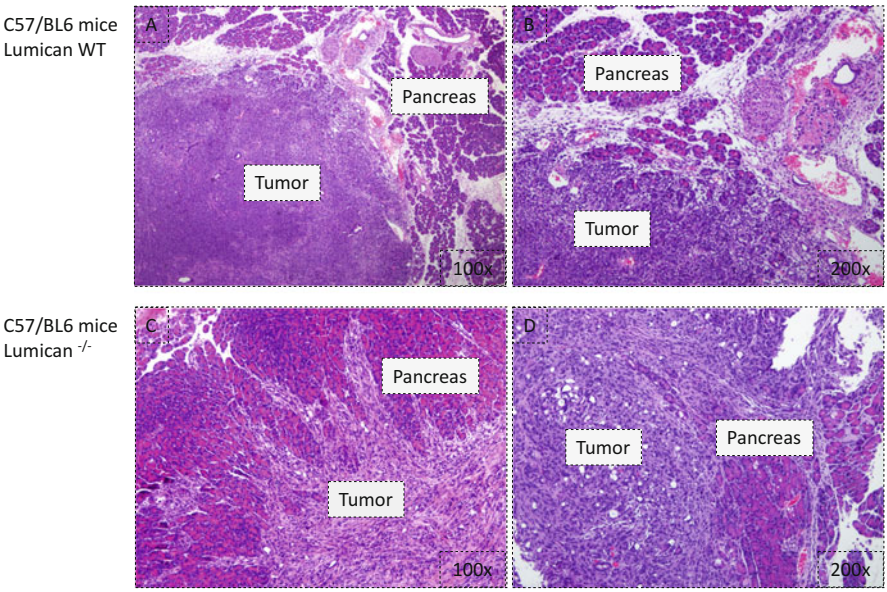

Fig. 3.1 H&E staining shows PanO2 tumor grown pattern in C57/BL6 wild-type (**a, b**) and lumican$^{-/-}$ (**c, d**) mice in orthotopic xenograft model

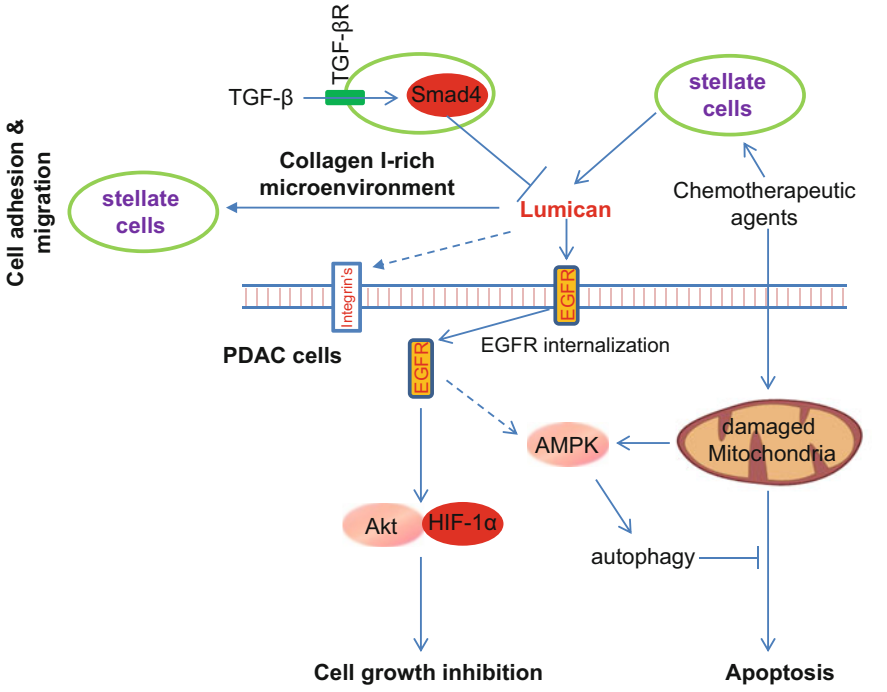

Fig. 3.2 A schematic of extracellular lumican function in pancreatic cancer. Figure adapted from Li et al. (2014, 2016)

3.3 Drug Delivery in Cancer and Its Association with Lumican Expression

A variety of barriers can prevent cancer drug delivery, not the least of which is the ECM in the tumor microenvironment (Sriraman et al. 2014). Achieving the necessary concentration of drugs within the tumor cells is particularly hindered by their inability to penetrate the tumor. This effect has been linked to the collagen content (Netti et al. 2000) and lack of proper vascularity (Folkman et al. 1989) in many of these tumors. Stromal lumican is typically identified within numerous human solid tumor malignancies (Naito 2005; Dolhnikoff et al. 1998; Qin et al. 2001; Baba et al. 2001; Onda et al. 2002; Ping Lu et al. 2002; Matsuda et al. 2008; Nikitovic et al. 2008a; Leygue et al. 1998; Ishiwata et al. 2007). While its expression has been observed in stromal and cancer tissue (Nikitovic et al. 2008b), this is not a consistent feature over all types of cancer. In addition, the differential expression of lumican based on tumor stage has also been noted (Panis et al. 2013). It is therefore important to consider lumican when considering the problem of solid tumor drug delivery.

Cancer has been described as an "over-healing" wound (Schafer and Werner 2008) in that there is a very often inflammatory response to the cancer cell growth that results in increased fibrosis. This desmoplastic reaction carries with it a number of side effects, which can build barriers to drug delivery. Of these obstacles, those most likely to have some connection with lumican are ECM density, inadequate vascularity, and increased tumor interstitial fluid pressure of many of these lesions.

3.3.1 Collagen Organization in Tumors

The ECM is a collection of extracellular molecules (including collagen, proteoglycans, etc.) that provide structural and biochemical support to the surrounding cells. It has been shown that a well-organized ECM impedes the progress of macromolecules through the tumor interstitium (Netti et al. 2000). Although as yet unproven, it is probable that lumican acts to organize and create an evenly spaced network of collagen fibrils within the TME. In so doing, this organized collagen could prevent distribution of therapy throughout the tissue. Investigations into drug distribution in lumican-negative tissues versus those with normal lumican are necessary to establish such a role in tumor dynamics. To counteract this obstacle, studies have discovered that collagenase pretreatment increases the penetration and distribution of therapy within solid tumors (Goodman et al. 2007). Matrix metalloproteases (MMPs) actually fulfill a similar role in terms of matrix degradation and proteolysis (Stetler-Stevenson and Yu 2001). Higher levels of MMP activity would result in increased drug delivery to tumors due to collagen matrix clearing. However, lumican has been shown to have MMP inhibitory activity (Pietraszek et al. 2014) and protects collagen from degradation (Geng et al. 2006), therefore theoretically

doubling its effectiveness in terms of creating a dense, organized ECM. However, it remains to be seen how the presence of lumican within a solid tumor ECM affects collagen organization, remodeling, and drug delivery.

3.3.2 VEGF and PDGF

Angiogenesis, and the associated increased levels of VEGF and platelet-derived growth factor (PDGF) in the TME (Kerbel 2008), is necessary for solid tumor progression beyond the earliest stages (Folkman et al. 1989). While VEGF and PDGF encourage new vessel growth and increase vessel permeability (Bates and Curry 1996; Harhaj et al. 2002), the delivery efficiency of tumor blood vessels is low. Additionally, the high interstitial pressure in the tumor forces the diffusion gradient into and not out of the vessels (Carmeliet and Jain 2000). Lumican inhibits angiogenesis (Sharma et al. 2013), specifically through the inhibition of VEGF (Albig et al. 2007). Another structurally similar SLRP, decorin, demonstrates PDGF-inhibiting activity (Baghy et al. 2013; Iozzo 1997). Both VEGF and PDGF have been reported to increase MMP and collagenase activity within the interstitium (Unemori et al. 1992; Sun et al. 2013), leading to rapid turnover and instability in ECM structures. One hypothesis about SLRPs is that they act to stabilize the vasculature and collagen matrix within the ECM of tumors. The normalization of tumor vasculature improves the delivery of cytotoxic therapy as seen in animal models (Carmeliet and Jain 2000), which opens the door for lumican as a therapeutic intervention to stabilize the TME.

3.4 Summary

Understanding all of the complex interactions between the tumor and its surrounding ECM is challenging; however, manipulating the ECM has proven to be an effective strategy to combat tumor progression and improve therapeutic delivery. Altogether, current evidence supports lumican as an antitumor molecule, although the importance of patient age, cancer site, and tumor stage should be taken into account when interpreting this data. In the proper context, however, lumican could represent a useful diagnostic and prognostic marker. Certainly, further studies are necessary to translate basic research on lumican into clinical application.

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Chapter 4
Versican: Role in Cancer Tumorigenesis

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Abstract Versican is an extracellular matrix proteoglycan that is expressed in a wide variety of cancers. Several cellular sources for versican have been identified in a multitude of cancers including tumor cells, stromal cells, myeloid cells, and lymphoid cells. Versican plays a role in five of the six hallmarks of cancer including proliferative signaling, evasion of growth suppressor signaling, promotion of tissue invasion and metastasis, angiogenesis, and resistance to cell death. Versican also interacts with growth factors and cytokines to modify their activity and involvement in the cancer response. The synthesis and accumulation of versican is regulated by similar pathways that regulate cancer progression, such as the canonical Wnt/β-catenin pathway and receptor tyrosine kinases. The expression and accumulation of versican are associated with poor prognosis, disease progression, metastasis, and chemoresistance. A detailed analysis of the role of versican in the disease course of leiomyosarcoma is provided here as an example of the importance of this extracellular matrix component in cancer pathogenesis. Collectively, our results and those from other groups suggest that versican could serve as a point of control in the management and treatment of many cancers.

Abbreviations

ADAMTS	*A d*isintegrin *a*nd *m*etalloproteinase with a *t*hrombo*s*pondin family
CAF	Cancer-associated fibroblast
CTLs	Cytotoxic T lymphocytes
DAMP	Danger-associated molecular pattern
ECM	Extracellular matrix
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
α-GAG	α-Glycosaminoglycan
β-GAG	β-Glycosaminoglycan

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HAS	Hyaluronan synthase
HYAL1	Hyaluronidase-1
LEFs	Lymphoid-enhancing factors
LMS	Leiomyosarcoma
LOX	Lysyl oxidase
MMP	Matrix metalloproteinase
PSCs	Pancreatic stellate cells
PSGL-1	P-selectin glycoprotein ligand-1
RHAMM	Hyaluronan-mediated motility receptor
αSMA^+	Alpha smooth muscle actin positive
TAMs	Tumor-associated macrophages
TCFs	T-cell factors
TGFβ	Transforming growth factor beta
TLR2	Toll-like receptor 2
TNFα	Tumor necrosis factor α
TSP1	Thrombospondin-1

4.1 Introduction

Versican is a large extracellular matrix (ECM) proteoglycan, named in recognition of its versatile modular structure (Fig. 4.1). Versican belongs to the hyaluronan-binding family of proteoglycans (hyalectins) whose other members include aggrecan (abundant in cartilage), brevican, and neurocan (nervous system proteoglycans). Versican expression is normally low in adult tissues but dramatically increases during development (Dutt et al. 2006; Perris et al. 1996), inflammatory disease (Cattaruzza et al. 2002; Wight 2002; Wight et al. 2014), and in a number of cancers (reviewed by Du et al. 2013; Ricciardelli et al. 2009; Theocharis et al. 2010). Furthermore, as will be discussed, versican is central to many of the hallmarks of cancer (Hanahan and Weinberg 2000) such as proliferative signaling, the evasion of growth suppressors, the promotion of tissue invasion and metastasis, angiogenesis, and resistance to cell death (Fig. 4.2). Versican also appears to be central to the more recently described hallmarks of cancer, which include immune surveillance evasion, immunomodulation, and tumor-promoting inflammation (Hanahan and Weinberg 2011). In this chapter, we will address (1) the sources of versican in cancer, (2) the structure and binding partners of versican and their expected function in cancer progression, (3) known regulators of versican expression implicated in cancer, and (4) proposed mechanisms of how versican regulates cell behaviors critical for tumor progression.

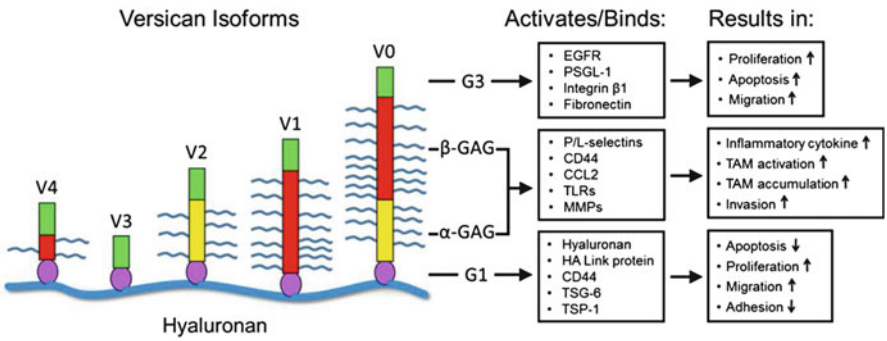

Fig. 4.1 Domain structures of versican isoforms and the importance of these interactions with various cell surface receptors and other ECM molecules. As a result of binding, charge-charge attraction and/or repulsion, bridging, or complex stabilization, versican, depending on its form, facilitates cancer progression

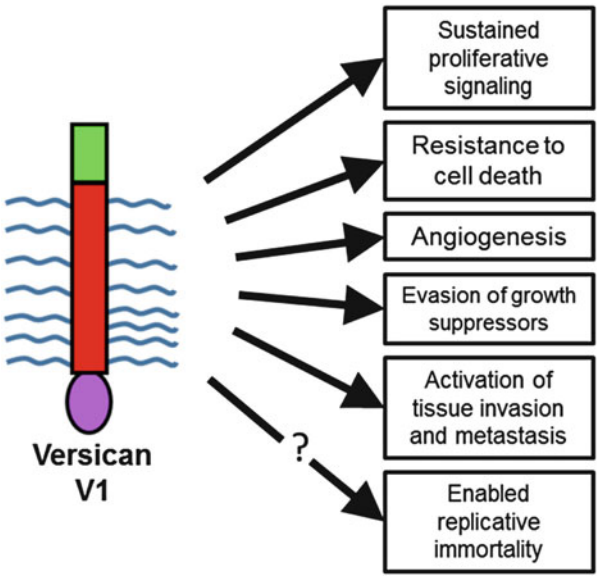

Fig. 4.2 Versican participates in at least five of the six hallmarks of cancer described originally by Hanahan and Weinberg (2000)

4.2 Source of Versican in Cancer

There are at least four major sources of versican in cancer: the tumor cells, tumor-associated stroma, tumor-associated myeloid cells, and tumor-infiltrating lymphoid cells. For example, tumor cells show an elevated expression of versican in lung carcinoma (Kim et al. 2009), ovarian cancer (Li et al. 2013), leiomyosarcoma

(LMS; Keire et al. 2014), hepatocellular carcinoma (Xia et al. 2014), colon carcinoma (Bogels et al. 2012), glioma (Hu et al. 2015), myeloma (Hope et al. 2014), and bladder cancer (Said and Theodorescu 2012). Amplified expression of versican in cancer cells is also observed in primary malignant and metastatic melanomas (Touab et al. 2002). This upregulation of versican by cancer cells themselves is significant for several reasons. In the case of Lewis lung and breast carcinomas, the presence of versican leads to an accumulation and activation of tumor-associated macrophages (TAMs) via toll-like receptor 2 (TLR2) and its co-receptors TLR6 and CD14 (Du et al. 2013; Grivennikov et al. 2010; Kim et al. 2009; Tang et al. 2015; Hope et al. 2016). Similarly, versican expressed by glioma cells promotes tumor expansion via TLR2 receptors on resident microglia/macrophages in the brain (Hu et al. 2015). There are multiple ramifications of versican-TLR2 engagement on myeloid cells. For example, the activation of microglial cells via TLR2 ligation with versican leads to the expression of MT1-MMPs and a cascade of proteases that characterize the spread and growth of glioma (Hu et al. 2015; Liu et al. 2015; Busek et al. 2016). Versican engagement of the TLR2 receptor complex also induces tissue necrosis factor α (TNFα) secretion by myeloid cells and thus strongly enhances tumor proliferation and metastatic growth. By another mechanism, in bladder cancer, elevated versican expression by cancer cells inhibits protective metastatic suppressor G-protein signaling genes, thus facilitating an aggressive cancer phenotype (Said and Theodorescu 2012). Moreover, versican release into the tumor microenvironment by the tumor cells may affect the ability of the immune system to mount an appropriate response. Versican binding of TLR2 on the surface of dendritic cells, which are a key link between innate and adaptive immunity (Steinman 2012), leads to a suppression of immune surveillance. Dendritic cells typically prime CD8+ T cells which results in the generation of cytotoxic T lymphocytes (CTLs); however, when versican binds to dendritic cells, IL-6 and IL-10 are released, leading to dysfunctional CTL activity, tumor growth, and metastatic progression (Tang et al. 2015). Collectively, these studies find a direct correlation between tumor versican expression and tumor grade. In addition, our experience with LMS indicates that the level of versican expression by tumor cells is directly associated with the aggressiveness and progression of the cancer (Keire et al. 2014).

In other cancers and their subtypes, stromal cells serve as a major source of versican, such as in cancers of the breast (de Lima et al. 2012; Kischel et al. 2010; Nara et al. 1997; Ricciardelli et al. 2002; Takahashi et al. 2012), colon (Iozzo 1995; Iozzo et al. 1982), ovaries (Yeung et al. 2013), and prostate (Ricciardelli et al. 1998; Sakko et al. 2001, 2003, 2007; True et al. 2009). Versican is typically not expressed in normal breast tissue, but with the onset of cancer, it is observed at low levels in ductal epithelial cells and at even higher levels in periductal lobular stroma (de Lima et al. 2012). In the case of breast cancer, it is likely that the overexpression of transforming growth factor beta (TGFβ) by ductal epithelial cells contributes to this pattern of strong stromal versican induction and cancer progression (Derynck et al. 1987; Van Bockstal et al. 2014) as versican expression is upregulated in response to TGFβ in a variety of cell types (Onken et al. 2014; Nikitovic et al. 2006; Schönherr et al. 1991; Kähäri et al. 1991). In a similar manner, versican was

identified as a key TGFβ-induced gene in cancer-associated fibroblasts (CAFs) associated with ovarian cancer progression (Yeung et al. 2013).

In some cancers, such as endometrial and cervical cancers, tumor and stromal cells display increased levels of versican (Kodama et al. 2007a, b). The combination of tumor and stromal expression of versican correlates with shortened disease-free survival and overall survival (Kodama et al. 2007b). Moreover, in cervical cancer, disease progression is marked by increased levels of versican in tumors, lymph node metastases, and in the lymph-vascular space (Kodama et al. 2007a). As has been identified in other cancers, elevated levels of versican expressed by cancer and stromal cells appear to enhance the activation of TAMs, which likely promotes cervical cancer progression, but the role of versican in this cancer has not been studied in great detail. Paradoxically, system-wide ($Vcan^{flox/flox}$) ablation of versican expression leads to a decrease in the number and density of CAFs in stroma as seen in a fibrosarcoma tumor model (Fanhchaksai et al. 2016). Notably, decreases in CAFs through alpha smooth muscle actin-positive (αSMA^{+}) myofibroblast ablation have been associated with immunosuppression, more aggressive tumor behavior, and a poorer prognosis in pancreatic cancer (Ozdemir et al. 2014). The problem with such experiments is the unintentional depletion of the fraction of αSMA^{+} cells which are homeostatic and the consequent loss of the natural compartmentalization of the organ matrix imparted by the tissue stroma. Other cells in the tissue stroma, such as the pancreatic stellate cells (PSCs), are in low abundance in the normal homeostatic pancreas (Omary et al. 2007); however, when activated, PSCs comprise a large portion of the desmoplastic pancreatic cancer matrix associated with pancreatic ductal adenocarcinoma progression and metastasis (Neesse et al. 2011). If it is determined that versican is produced by PSCs, this is another mechanism whereby versican accumulation in the stroma could lead to cancer progression. Versican, for example, is implicated in interfering with T cell-mediated tumor destruction by displacing T cells from the appropriate tumor target (Joyce and Fearon 2015). Whether versican has dual functions in regulating the behavior of tumor cells and stromal cells to impact tumor progression and immunosuppression is not yet clear and requires further study.

Myeloid cells are also a major source of versican under inflammatory (Gao et al. 2012a; Chang et al. 2012, 2014) and hypoxic conditions (Asplund et al. 2010, 2011; Sotoodehnejadnematalahi et al. 2015; Wang et al. 2015; Zhang et al. 2012). Consistently, in breast cancer, versican derived from myeloid cells is critical in promoting tumor metastasis (Gao et al. 2012b). Using a murine model of spontaneous breast cancer, Gao and colleagues found that versican expressed by CD11b+ $Ly6C^{high}$ myeloid cells promotes lung metastasis in a TGFβ-dependent manner (Gao et al. 2012a). Intriguingly, co-culture of myeloid cells with bladder carcinoma cells results in an upregulation of versican in the myeloid cells, suggesting that the source of versican in cancerous tumors includes myeloid cells associated with the tumor (Said and Theodorescu 2012). Such co-sourcing of versican from both the myeloid cells (e.g., TAMs and myeloid-derived suppressor cells) and from the cancer cells serves to exacerbate the spread of the cancer and is thought to facilitate cancer progression (Gutmann 2015; Senda et al. 2016; Said et al. 2012). Such studies highlight potential crosstalk created by versican among different cell types

that may provide key links to cancer initiation, promotion, immunosuppression, and metastatic progression.

4.3 The Role of Versican in Cancer

There are at least five naturally occurring versican isoforms that have been identified and characterized. The isoforms, designated V0, V1, V2, and V3, are generated by alternatively splicing the central α-glycosaminoglycan (α-GAG) and β-glycosaminoglycan (β-GAG) domains. Versican isoforms V0 and V1 are the predominant isoforms produced by adult mesenchymal cells with V1 as the most abundant form (Kischel et al. 2010; Wight et al. 2014) and the most highly expressed in late-stage or metastatic cancer. V2 is primarily expressed in neural tissue and not typically by other tissues, and V3 is variably expressed in a number of tissues but at comparably lower levels than the other isoforms (Lemire et al. 1999). Moreover, it was discovered that at least in breast cancer, a unique fifth versican splice variant termed V4 is expressed (Kischel et al. 2010). Versican V4 arises from a splice variation of exon 8, resulting in a truncated or shortened β-GAG domain (Fig. 4.1).

Common to these splice variants are the N- and C-terminal ends or G1 and G3 domains, respectively. The G1 domain of versican, which contains a hyaluronan-binding region, mediates cell proliferation, adhesion, and migration (Yang et al. 1999), while the G3 domain is involved in cell phenotype control through its association with integrins (Wu et al. 2004, 2005), microfibrilar fibulins (Miosge et al. 1998), and epidermal growth factor (EGF) receptors (Xiang et al. 2006). Sakko et al. have shown that an increase in versican expression in the ECM facilitates local tumor invasion and metastasis by decreasing cell-ECM adhesion (Sakko et al. 2003). One of the mechanisms by which versican affects ovarian tumors is through binding to cell surface CD44 (Ween et al. 2011). This in turn activates signaling pathways such as JNK and NF-κB and thus enhances cell migration and tumor progression through the production of tumorigenic proteins, such as hyaluronan-mediated motility receptor (RHAMM) and matrix metalloproteinase (MMP)-9 (Yeung et al. 2013). The G1 domain of versican also binds to thrombospondin-1 (TSP1; Kuznetsova et al. 2006). The concurrent upregulation of TSP1 and versican is reported in stromal cells of human breast carcinomas (Brown et al. 1999). Thus, the interaction of versican with various ECM components via the G1 or G3 domains is likely to regulate the stromal responses that are critical to modulating cancer progression (Bhowmick et al. 2004). In general, the G1 domain of versican is thought to stimulate proliferation by destabilizing cell adhesion (Yang et al. 1999), while the G3 domain mediates proliferation through EGF-like domain interaction with EGF receptors (Ang et al. 1999; Du et al. 2010; Zhang et al. 1998, 1999). In support of this, a corresponding relationship of high versican expression to tumor growth is observed in gliomas, breast, prostate, gastric, pancreatic carcinomas, and uterine sarcomas (Cai et al.

2013; Keire et al. 2014; Nara et al. 1997; Onken et al. 2014; Shen et al. 2015; Skandalis et al. 2006a; Wade et al. 2013).

Between the terminal G1 and G3 domains of versican are alternatively spliced α-GAG and β-GAG attachment domains important to versican biology (Wu et al. 2005; Wight 2002). The GAG chains are known to interact with inflammatory mediators and are key to versican's role in the progression of the cancer phenotype described by Hanahan and Weinberg (2000, 2011; Fig. 4.2). Versican interacts with inflammatory mediators via its chondroitin sulfate chains, including CCL2/MCP1, CD44, P-selectin glycoprotein ligand-1 (PSGL-1), TLR2, and MMPs (Hirose et al. 2001; Malla et al. 2013; Wang et al. 2009; Wu et al. 2005). In addition, the interaction of versican with hyaluronan via its G1 domain plays a significant role in the ability of cancer cells to migrate, adhere, proliferate, and interact with the surrounding ECM and immune system (Evanko et al. 2012; Frey et al. 2013; Keire et al. 2014; Toole et al. 2002; Wight et al. 2014). Hyaluronan is made entirely of repeating disaccharide (D-glucuronic acid β-1,3-*N*-acetylglucosamine-β-1,4) units and is synthesized by three related hyaluronan synthases (HAS1, HAS2, and HAS3; Toole et al. 2002).

Significantly, versican and hyaluronan interact to form large multimolecular weight aggregates around cells, which accumulate in various types of tumors and are associated with tumor progression, including tumors of the prostate (Bharadwaj et al. 2007; Ricciardelli et al. 2007), breast (Koyama et al. 2007; Suwiwat et al. 2004), bone (Nikitovic et al. 2006), lung (Pirinen et al. 2005), cartilage (Romeo et al. 2007), skin (Karvinen et al. 2003; Papakonstantinou et al. 2003; Touab et al. 2003), brain (LaPierre et al. 2007), pancreas (Skandalis et al. 2006a), cervix (Kodama et al. 2007a), uvea (Folberg et al. 2006), larynx (Skandalis et al. 2006b; Skandalis et al. 2004), mouth (Pukkila et al. 2007), testis (Labropoulou et al. 2006), and ovaries (Ricciardelli and Rodgers 2006; Ween et al. 2011). The expression of hyaluronan and versican in such a wide variety of cancers suggests an active role for these molecules in tumor development.

Versican and hyaluronan are ECM components that are at the center of angiogenesis (Du et al. 2013; Feinberg and Beebe 1983; Fu et al. 2011; Montesano et al. 1996; Rivera et al. 2011; Rooney et al. 1993, 1995; Slevin et al. 2007, 2009; West et al. 1985; West and Kumar 1989; Zheng et al. 2004b). Angiogenesis is a normal and vital process in development, wound healing, and cancer and, in part, occurs in matrices enriched in versican and hyaluronan. Versican levels in the tumor microenvironment have been shown to positively correlate with the number of microvessels in the tumor stroma of ovarian and testicular germ cell tumors (Ghosh et al. 2010; Labropoulou et al. 2006). We found that human stromal stem cells can regulate the angiogenic phenotype of endothelial cells by modulating the formation of provisional matrices enriched in versican and hyaluronan (Kreutziger et al. 2011). In addition, we found that different clonal stromal stem cell types support endothelial network formation in vitro with varying degrees of effectiveness. Stromal stem cells that produce elevated levels of versican formed a more extensive vascular network when co-cultured with vascular endothelial cells. Furthermore, patches containing these pro-angiogenic cells, when transplanted onto

uninjured athymic rat hearts, developed 50-fold more vessels than stromal cells with low versican expression (Kreutziger et al. 2011). Versican is actively processed during the early stages of VEGF-A-induced pathological angiogenesis (Fu et al. 2011). These observations plus the fact that the tumor stroma contains provisional matrix components such as fibrin, fibronectin, and hyaluronan (Dvorak 1986, 2002, 2015) highlight the importance of this specialized ECM in the pathogenesis of cancer. Dvorak first postulated that "tumors are wounds that do not heal" (Dvorak 1986) due to their high content of provisional ECM components such as versican. In angiogenic models, the increased expression of versican is often accompanied by an increased expression of hyaluronan (Koyama et al. 2007; Nara et al. 1997). Hyaluronan and fragments of hyaluronan play a key role in new blood vessel formation, affecting the behavior of endothelial cells (Feinberg and Beebe 1983; Montesano et al. 1996; Rooney et al. 1993, 1995; Slevin et al. 2007, 2009; West et al. 1985; West and Kumar 1989).

An ECM enriched in hyaluronan and versican has also been shown to promote myeloid cell adhesion and retention (de la Motte et al. 2003; Potter-Perigo et al. 2010; Wilkinson et al. 2006), which was recently acknowledged as an important aspect of tumor progression by its recruiting myeloid-derived suppressor cells and TAMs (see reviews by Kitamura et al. 2015; Marvel and Gabrilovich 2015; Shalapour and Karin 2015). The interaction between versican and myeloid cell surface receptors such as PSGL-1 and TLR2 further induces macrophage aggregation (Zheng et al. 2004a) and the expression of cytokines and MMPs (Bogels et al. 2012; Hu et al. 2015; Kim et al. 2009; Wang et al. 2009; Zhang et al. 2012). These findings indicate that chondroitin sulfate-bearing isoforms of versican promote leukocyte accumulation and activation. Our studies demonstrated that blocking versican accumulation by a blocking antibody or regulating versican synthesis inhibits the adhesion of monocytes/macrophages to the ECM (Kang et al. 2014; Potter-Perigo et al. 2010). The mechanism of versican-dependent monocyte/macrophage adhesion is achieved by attenuating the activation of NF-κB p65 as well as a number of NF-κB-responsive pro-inflammatory molecules, which promote leukocyte adhesion and accumulation including VCAM1, ICAM1, CCL2 (MCP1), and CXCL1 (Kang et al. 2014, 2015). These molecules have also been shown to be critical in promoting metastasis (Kitamura et al. 2015). Recent studies have established that versican is a danger-associated molecular pattern (DAMP) molecule that activates TLR2 on macrophages leading to the production of inflammatory cytokines such as TNFα and IL-6, which significantly increase invasion and metastatic growth in many cancers including ovarian and bladder cancer, Lewis lung carcinoma, myeloma, and glioma (Bogels et al. 2012; Hope et al. 2014; Hu et al. 2015; Kim et al. 2009; Said et al. 2012; Wang et al. 2009).

Numerous studies have shown that increased levels of versican and hyaluronan correlate with elevated metastatic potential and poor disease prognosis (Kim et al. 2009; Labropoulou et al. 2006; Nikitovic et al. 2006). Versican stimulates inflammatory cytokine production by bone marrow mononuclear cells, thus facilitating metastasis (Kim et al. 2009). Versican, through its binding to TLR2 and adhesion molecules expressed by inflammatory cells, leads to the activation of those cells and

the expression of inflammatory modulating cytokines such as TNFα, IL-1β, and IL-6 (Kim et al. 2009). These cytokines contribute to the establishment of an inflammatory cancer cell microenvironment favoring proliferation, tissue invasion, and metastasis. Furthermore, in prostate cancer, versican's binding partner hyaluronan and its fragments bind to TLR2, synergizing the activation of monocytes to macrophages and the production of inflammatory cytokines (Hu et al. 2015; Lokeshwar et al. 2005).

The inhibition of hyaluronan and versican production has been associated with decreased cancer progression. For example, antisense inhibition of HAS2 in osteosarcoma cells inhibits hyaluronan retention and tumorigenicity (Nishida et al. 2005). Moreover, silencing the gene for HAS2 using RNA interference (RNAi)-mediated suppression leads to a less aggressive phenotype of breast tumor cells (Li et al. 2007). Versican also appears to synergize with hyaluronan to drive cell proliferation (Keire et al. 2014).

4.4 The Regulation of Versican Expression in Cancer

A number of key signal transduction pathways critical for tumorigenesis have been identified as regulators of versican expression. Enhanced versican levels in ovarian cancer leads to the subsequent activation of both the JAK/STAT and PI3-kinase/AKT pathways (Carvalho et al. 2003; Ricciardelli and Rodgers 2006; Ween et al. 2011). In the process of ovulation, an increase in luteinizing hormone leads to a dramatic increase in versican and other ECM components in the cumulus-oocyte complex (Russell et al. 2003). Both G protein-coupled receptors and tyrosine kinase proteins are activated with the induction of ovulation by luteinizing hormone (Carvalho et al. 2003). Expression of versican rises and falls during the ovarian cycle; however, in ovarian carcinoma, versican is constitutively and continuously expressed at relatively high levels. This dysregulation involves specific signaling pathways such as JAK/STAT and PI3-kinase/AKT. It is known that the activation of AKT leads to increases in β-catenin signaling. Importantly, the canonical Wnt/β-catenin pathway, which is critical in early embryogenesis, cell differentiation, and neoplasms (Huang and He 2008; Korswagen and Clevers 1999; Taipale and Beachy 2001), is a primary driver of versican expression (Rahmani et al. 2006). The accumulation of β-catenin and subsequent formation of a complex with T-cell factors (TCFs) or lymphoid-enhancing factors (LEFs) on the versican promoter leads to increased versican expression. Interestingly, the tumor suppressor gene, p53, promotes versican expression, especially postradiation (Yoon et al. 2002). Often p53 is mutated in cancer, so as the cell attempts to control rampant cell cycling, even more p53 is produced in advanced or high-grade tumors (Mattioni et al. 2015). Other factors such as promotor methylation and microRNA expression may also control versican expression levels. For example, key microRNA sequences have been recently shown to impact versican expression and play a role in benign versus metastatic tumor states (Li et al. 2014). In addition,

versicanases, such as *a d*isintegrin *a*nd *m*etalloproteinase with *t*hrombo*s*pondin motifs (ADAMTS-5), expressed by T cells, degrade versican thus controlling versican levels (McMahon et al. 2016) and the tumor-directed CTL response described earlier.

4.5 Versican as a Diagnostic and Prognostic Marker in Cancer

Versican expression is diagnostically associated with a poor prognosis, disease progression, metastasis, and chemoresistance in cancers. Elevated levels of versican, hyaluronan, and CD44 are all associated with the poor prognosis of ovarian cancers (Ricciardelli and Rodgers 2006). Furthermore, high stromal versican staining is associated with reduced 5-year survival rates of ovarian cancer patients (44 versus 32%; Voutilainen et al. 2003). Versican is significantly upregulated in chemoresistant ovarian cancer when compared to chemosensitive ovarian cancer (Pan et al. 2009). For example, in primary oral squamous cell carcinoma, increases in stromal versican correlate with both an increased risk for disease recurrence and shortened patient survival (Pukkila et al. 2007). In colon cancer, versican, biglycan, collagen1A1, and sulfatase1/sulfatase2 expression are identified as potential tumor microenvironment biomarkers and/or targets for diagnostics and treatment (Suhovskih et al. 2015). In addition, versican is identified as significantly upregulated along with β-catenin, β1 integrin, and focal adhesion kinase (FAK) in the disease course of multiple myeloma, which is a malignancy of B cells characterized by the proliferation and dissemination of malignant plasma cells from the bone marrow (Gupta et al. 2015). However, the prognostic role of versican is tissue specific. For example, in pharyngeal squamous cell carcinoma, versican expression in the primary tumor is not an independent prognostic factor, although a signature of significantly higher versican staining in the draining lymph nodes of the tumor is observed (Pukkila et al. 2004). Versican has been identified in a number of other cancers as having some diagnostic and prognostic value as well (Sluiter et al. 2016; Kobayashi et al. 2015; Driessen et al. 2016; Ju et al. 2010).

4.6 Versican in LMS: Our Experience

Significantly greater amounts of versican are expressed and accumulate in LMS compared to benign leiomyomas and normal healthy tissue (Keire et al. 2014; Fig. 4.3, panels a–e). Supporting microarray analyses of 80 LMS tumors and 24 leiomyomas showed a significant increase in versican mRNA in LMS versus benign leiomyomas. Such findings indicate that versican may play a role in mediating the aggressiveness of LMS tumors compared to leiomyomas. We also

demonstrated that inhibiting versican synthesis in LMS cells using versican-directed siRNA reduced their proliferation and migration in vitro (Fig. 4.3, panels f and g). LMS cells form extensive pericellular matrices enriched in versican and hyaluronan when grown in tissue culture, and inhibiting versican synthesis in these cells dramatically reduced the thickness of their pericellular matrices (Fig. 4.3, panels h and i). Adding versican back to these cells restored both the thickness of their pericellular coats (Fig. 4.3j) and heightened their proliferative rate (Fig. 4.3k). Nude mice injected with LMS cells stably expressing versican shRNA developed tumors with lower volumes and mitotic indices compared to mice injected with control LMS cells (Fig. 4.4, panels a and b). The manner in which versican and hyaluronan are thought to influence cell phenotype is shown diagrammatically in Fig. 4.5. Collectively, these results provide a potential strategy to control versican expression in LMS. Constitutive siRNA knockdown of versican in LMS cells resulted in increased expression of tropoelastin in vitro as assessed by qRT-PCR, immunohistochemistry, and Western blot analyses (Keire et al. 2016). Desmosine analysis, a marker for elastin synthesis and maturation, confirmed a 70% increase in elastin over LMS controls. Microarray analysis identified significant changes in 270 genes expressed in versican knockdown cells, a subset of which were selected for later validation by TaqMan low-density microarray. Within the set of 96 genes analyzed by TaqMan low-density array, tropoelastin was significantly upregulated as were elastin-associated genes that included fibulin-1, fibulin-5, and lysyl oxidase (LOX). LOX is an enzyme that initiates the cross-linking of collagen and elastin. In addition to cross-linking ECM proteins, LOX appears to play a role in tumor suppression (Bouez et al. 2006). Fibulin-5 is an elastin-associated protein expressed by endothelial cells and fibroblasts. The overexpression of fibulin-5 in endothelial cells results in reduced proliferation (Preis et al. 2006), while fibulin-5-expressing hepatocellular carcinoma cells exhibit decreased migration and invasion by downregulating the expression of the elastin-degrading enzyme, MMP-7 (Tu et al. 2014). Gene array and cell culture studies are further supported by in vivo studies, where versican siRNA LMS tumor cells injected into nude mice deposit significantly more elastic fibers than do control LMS cells (Keire et al. 2016). Collectively, in vitro and in vivo results suggest an important role for versican in regulating tumorigenesis and tissue homeostasis through the regulation of homeostatic molecules such as elastin.

In addition, we have found that the downregulation of versican leads to significant changes in the expression of a number of ECM proteolytic genes in LMS cells (Keire et al. 2016). For example, with the downregulation of versican, significant increases in MMP-12, ADAMTS-9, ADAMTS-20, and hyaluronidase-1 (HYAL1) levels are accompanied by substantial decreases in HYAL2, ADAMTS-4, and MMP-7. These changes are consistent with a less aggressive or benign cancer phenotype. For example, antisense-mediated suppression of HYAL2 inhibits breast cancer tumorigenesis and progression (Udabage et al. 2005). The overexpression of MMP-7 and MMP-9 is implicated in the invasion and metastasis of colorectal cancer (Woo et al. 2007) as well as in breast cancer (Vizoso et al. 2007), while MMP-12 overexpression is associated with increased survival and decreased

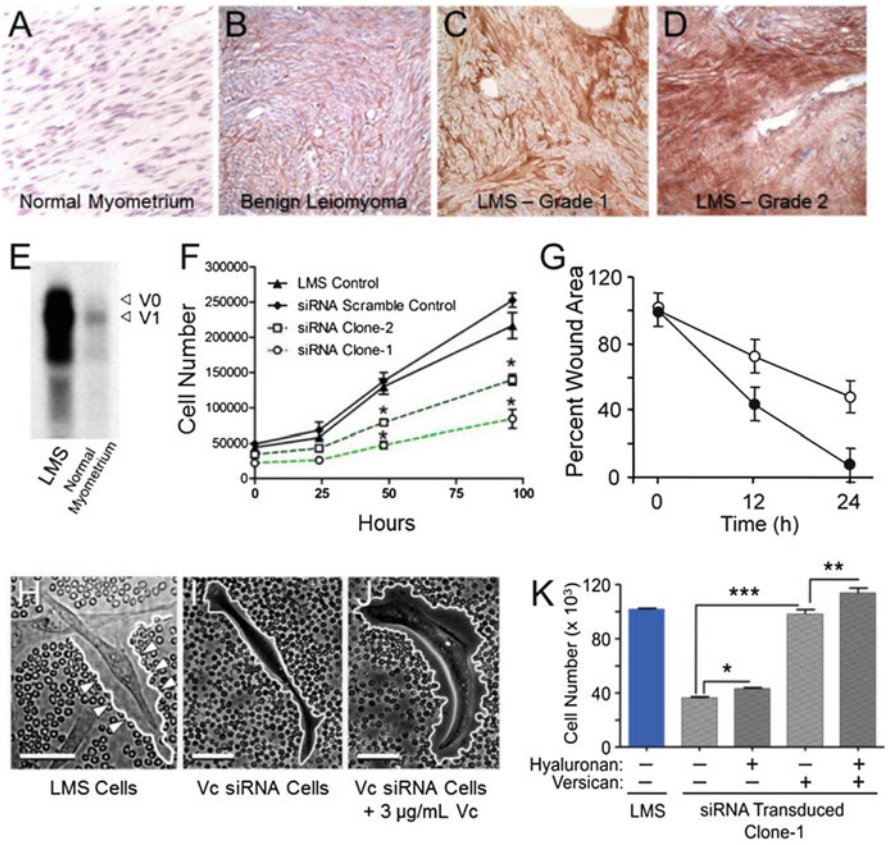

Fig. 4.3 Versican is highly expressed in clinical samples of leiomyosarcoma compared to normal tissue and benign tumors, and downregulation of versican dramatically changes cell phenotype. Normal human myometrium (**a**) stained for versican shows no staining, compared to a representative, benign leiomyoma tumor (**b**), which shows a greater amount of versican (*brown*) staining, but less than grades 1 (**c**) and 2 (**d**) LMS, which have extensive immunostaining. Northern blot analyses show increased versican in the LMS tumor compared to control (**e**). Cell proliferation assays indicate that the LMS/WT (*filled triangles*) and LMS/siRNA Scramble (*filled circles*) control cells divide and proliferate at a significantly higher rate than the two different versican siRNA LMS cell clones (*open squares* and *open circles*) (**f**). In a scratch wound cell migration assay (**g**), the migration of LMS cells (*filled up-pointing triangles*) was significantly greater (*single asterisks*, $p < 0.05$) at 12 and 24 h than that of LMS cells transduced with versican siRNA (*open circles*) ($n = 4$). LMS smooth muscle cells in culture treated with fixed red blood cells to image the pericellular matrix (**h–j**). The LMS cells exhibit extensive pericellular coats (**h**), while the LMS cells in which versican expression has been inhibited lack extensive cellular coats (**i**). LMS pericellular coat 24 h after adding back versican display extensive cell coats (**j**). *Arrowheads* (*white triangles*) and *solid white lines* mark the pericellular boundaries. *Scale bars* 50 µm. Large molecular weight hyaluronan by itself does not restore the proliferative profile of LMS/siRNA Vc cells to LMS/WT levels but does with the addition of purified versican (**k**). Although there is a significant increase in cell proliferation with the addition of hyaluronan (30 µg/ml; *single asterisks*, $p < 0.015$), the increase due to the addition of versican at nanogram levels is significantly greater (*triple asterisks*, $p < 0.0001$), and near complete restoration (96.6%) of the native LMS cell proliferative rate is achieved at 100 µg/ml versican. The difference between versican alone and versican *plus sign* large hyaluronan is significant (*double asterisks*, $p < 0.004$), suggesting an

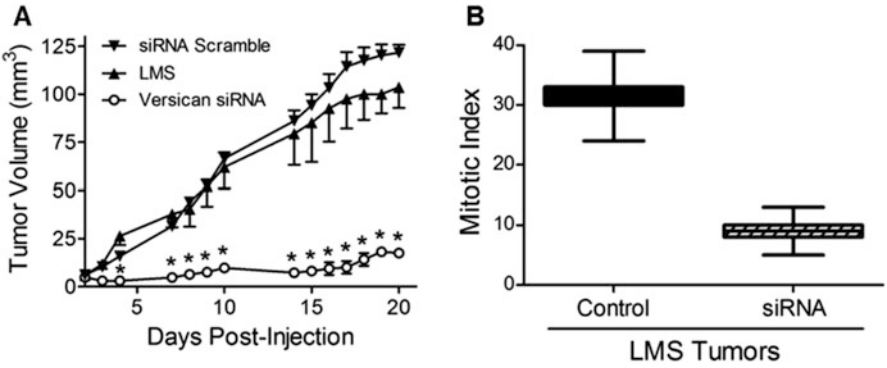

Fig. 4.4 Tumor growth in a mouse model of LMS using LMS cells treated or not treated with siRNA to versican. This figure shows (**a**) reduced tumor growth in the animals receiving siRNA versican LMS cells and (**b**) the mitotic index (MI) of LMS control versus LMS/siRNA Vc tumors. The *box graph* in (**b**) depicts median MI ± SD, and *error bars* show the minimum and maximum range of mitotic figures per 10 400× fields ($n = 15$). This figure was originally published in the *Journal of Biological Chemistry*. Keire PA, Bressler SL, Lemire JM, Edris B, Rubin BP, Rahmani M, McManus BM, van de Rijn M, Wight TN. A role for versican in the development of leiomyosarcoma. *J Biol Chem*. 2014; 289:34089–34103. © the American Society for Biochemistry and Molecular Biology

metastasis of colorectal cancers (Zucker and Vacirca 2004). Versican is a substrate of ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-9, and ADAMTS-20 (Stanton et al. 2011), and when versican is degraded, it is associated with vascular smooth muscle cell death in vivo (Kenagy et al. 2009). Interestingly, the gene for ADAMTS-9 is localized to chromosome 3p14.3-p14.2, an area known to be lost in hereditary renal tumors and esophageal cancer development (Lo et al. 2007). Furthermore, ADAMTS-9 and ADAMTS-20 expression suppresses esophageal and nasopharyngeal carcinoma tumor formation (Lo et al. 2010). This suggests that protease-specific versican degradation products may react differently in different tissues. For example, our research shows that the downregulation of versican leads to a decrease in the ECM-degrading proteases ADAMTS-4 and ADAMTS-5 which are highly expressed in human glioblastomas (Held-Feindt et al. 2006), whereas ADAMTS-9 and ADAMTS-20 are upregulated and may be homeostatic (Keire et al. 2016). Thus, versican may influence the phenotype of every cell directly and indirectly through the modulation of its ECM interactive partners and matrix modulatory enzymes.

Fig. 4.3 (continued) additive or synergistic effect between hyaluronan and versican on cell proliferation. This figure is adapted from research originally published in the *Journal of Biological Chemistry*. Keire PA, Bressler SL, Lemire JM, Edris B, Rubin BP, Rahmani M, McManus BM, van de Rijn M, Wight TN. A role for versican in the development of leiomyosarcoma. J Biol Chem. 2014; 289:34089–34103. © the American Society for Biochemistry and Molecular Biology

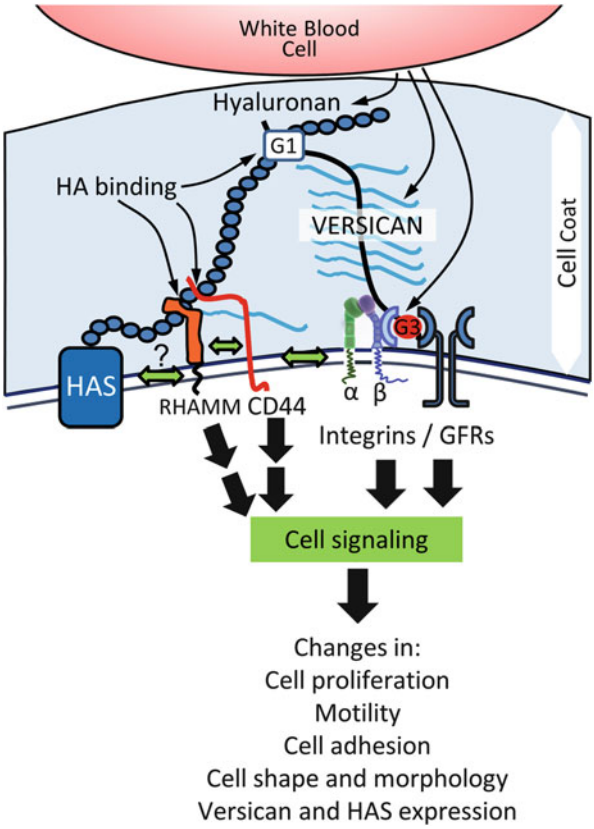

Fig. 4.5 Schematic diagram details the described interplay between versican and hyaluronan and how this interaction transduces changes in cell phenotype observed in cancer. Through its G3 domain, versican binds to and activates growth factor receptors and integrins leading to downstream cell signaling. The G3 and α- and β-GAG domains of versican bind white blood cells (neutrophils, eosinophils, basophils, T cells, B cells, NK cells, and monocytes) through their PSGL-1 cell surface receptors leading to downstream signaling and phenotypic changes in those cells. The HAS enzyme embedded in the plasma membrane synthesizes hyaluronan. The G1 domain of versican then interacts strongly with the emerging hyaluronan chains leading to cell surface localization. This in turn leads to interaction and activation of CD44, RHAMM, TLRs, MMPs, and other cell surface proteins. *RHAMM* receptor for hyaluronan-mediated motility; *GFRs* growth factor receptors; *HA* hyaluronan; *HAS* hyaluronan synthase

4.7 Conclusions

Versican, true to its name, is a versatile molecule of many functional roles in cell and tumor biology. It plays a significant role in five of the six hallmarks of cancer originally described by Hanahan and Weinberg (2000). Versican supports sustained cell proliferation, chemoresistance, the evasion of growth suppression, tissue invasion and metastasis, angiogenesis, and apoptotic resistance (Fig. 4.2). The role of

versican in cancer progression involves both its impact on cancer cell phenotype (proliferation, migration, metastasis) and how it impacts the surrounding microenvironment and the ability of the immune system to identify and remove cancerous cells. In light of this, not only can versican be used as a diagnostic or prognostic marker in a wide variety of cancers, it may also serve as a potential therapeutic target for cancer therapies.

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Chapter 5
Engineering Advanced Models of the Glioblastoma Microenvironment Using Biomaterials

Andrew Rape and Sanjay Kumar

Abstract Glioblastoma (GBM) is the most common and deadly primary brain cancer. Patients diagnosed with GBM have a mean survival time of only 21 months, despite an intense push over the past several decades to dissect underlying mechanisms and develop new therapies. Whereas discovery efforts related to GBM have traditionally focused on cell-intrinsic factors, such as genetic and epigenetic lesions, it has more recently become clear that cell-extrinsic factors within the tumor microenvironment play important pathogenic roles as well. More surprisingly, physical aspects of the microenvironment, including tissue structure and mechanics, can regulate signaling events that contribute to dysplasia, invasion, and metastasis. This chapter will describe the basic biology of physical microenvironmental regulation of the GBM, with a focus on the extracellular matrix. We will also describe how components of the physical microenvironment can be recapitulated using biomaterials technology and how these new platforms can contribute to next-generation culture systems for discovery and screening.

5.1 Introduction

Glioblastoma (GBM) is the most common primary brain cancer, accounting for approximately 54% of all the brain tumors in the United States (Agnihotri et al. 2013). In addition to its high prevalence, GBM is also the most aggressive and lethal brain cancer, leading to death an average of 21 months from the time of diagnosis. Unfortunately given the severity of the diagnosis, there is currently no

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definitive treatment for patients afflicted with this disease. This is often attributed to the diffuse nature of the tumor, in which a primary tumor spreads outward from its point of origin, causing tumor cells to occupy large tracts of brain tissue, making complete surgical resection impractical if not impossible in most cases (Louis et al. 2007). This feature of GBM has long been observed and recognized as a critical bottleneck to successful therapy. It was most poignantly demonstrated by the neurosurgeon Dr. Walter Dandy in the 1920s, when he took the extreme step of removing an entire hemisphere of a brain affected with GBM in comatose patients only to see the tumor return post-resection (Dandy 1928).

The standard treatment of primary GBM consists of an intensive combination of surgical resection, radiotherapy, and a variety of traditional and directed chemotherapies (most notably, temozolomide) in an effort to ablate the tumor and prevent recurrence. Even given this extreme course of treatment, tumor recurrence occurs in approximately 90% of patients (Stupp et al. 2005; Berens and Giese 1999). The failure of current treatments is increasingly attributed to the cellular, genetic, and molecular heterogeneity of GBM tumors that appear similarly in clinical and histopathological presentation. This implies that GBM is not a single, unified disease but instead a collection of a few, if not many, distinct diseases that share clinical features. A comprehensive transcriptomic analysis from the Cancer Genome Atlas has led to a classification scheme involving four distinct genetic subtypes of GBM: classical, mesenchymal, proneural, and neural (McLendon et al. 2008; Verhaak et al. 2010). In addition to the genetic variability between patients in different tumors, there is also substantial intratumor heterogeneity, as exemplified by the emerging paradigm that tumor progression is driven by a potentially rare subset of "tumor initiating cells" (a.k.a. cancer stem cells) that share many of the genetic and phenotypic hallmarks of neural stem cells (Altaner 2008). Adding to this complexity, cells can transdifferentiate, spontaneously or under the influence of specific differentiation drivers, to pro-tumorigenic cell types. For example, tumor cells have been observed to transdifferentiate into endothelial cells and compete with host endothelial cells to populate tumor vasculature (Soda et al. 2011). Because GBMs represent myriad and unique genetic and molecular configurations, conventional treatments targeting aberrant genes or signaling pathways would only be expected to influence the subset of cells that fit the genetic framework targeted by the drug. Furthermore, recent high-resolution in vivo imaging efforts have suggested that GBM tumor cells can physically connect and exchange material over very long distances with membrane microtubes, the presence of which correlates with radioresistance. Thus, GBMs may represent an "organ within an organ" capable of a coordinated response to therapy (Osswald et al. 2015).

Given the spatial and temporal complexity of GBM, scientists have been increasingly searching for clinical targets that are shared by large percentages of GBM subtypes. Instead of focusing on intracellular signaling targets, new drugs aim to manipulate the extracellular environment as a means to harness cells even with substantial genetic, epigenetic, and molecular abnormalities. These extracellular regulatory components, collectively known as the microenvironment, include

the extracellular matrix (ECM), stromal and other nontumor cells near or within the tumor, and soluble and ECM-bound signals. In particular, targeting the contributions of the physical microenvironment, such as normalizing the mechanical properties of tumor cells and their environment, has drawn considerable interest as a therapeutic target. In the next few paragraphs, we will describe how each of these components regulates GBM progression (reviewed extensively in Charles et al. 2011; Payne and Huang 2013; Ulrich and Kumar 2011).

5.2 The Role of the Microenvironment in GBM Malignancy

5.2.1 ECM

Conventionally thought to serve as simply a passive scaffold to hold tumor and tumor-associated cells in the proper location and orientation, an extensive body of research has made it clear that the ECM can actively drive tumor progression in most tumor types, especially in GBM. Aberrations in both the ECM components of the microenvironment and of cellular receptors can both contribute to this phenomenon. The most abundant component of the ECM in the brain is the polysaccharide hyaluronic acid (HA), a glycosaminoglycan made up of repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine (Laurent and Fraser 1992; Toole 2004). The overproduction of HA, which is observed in most GBM tumors, is associated with cell proliferation and diffuse invasion away from the primary tumor. Despite the common association of HA overproduction with GBM, it remains unclear whether HA differentially regulates the various GBM subtypes or how this additional HA interacts with tumor/stromal cell-derived matrix to promote invasion. Cells engage HA through a set of transmembrane receptors including CD44, whose overabundance is also associated with cell invasion and growth (Delpech et al. 1993; Wiranowska et al. 2010; Ariza et al. 1995). CD44 expression is enriched at the tumor margin, suggesting that CD44 may facilitate adhesion to HA within normal brain parenchyma. In addition to its implied role in cell migration, CD44-HA ligation enhances pro-tumorigenic signaling inside tumor cells such as through the Rho family of small GTPases and PI3 kinase, which affect cell motility and proliferation, respectively (Herishanu et al. 2011). CD44 engagement of osteopontin, a matrix sialoprotein, was recently shown to induce cleavage of CD44 and trafficking of the intracellular domain to the nucleus, where it triggers tumor stem cell-like behaviors (Pietras et al. 2014).

Although HA is the most abundant ECM constituent around tumor cells, there are many other ECM molecules present in the GBM microenvironment. Particularly consequential to tumor development is the presence of tumor-associated vasculature and its specific associated microenvironment, which contains collagen IV, collagen V, fibronectin, and laminin (Giese and Westphal 1996; Knott et al.

1998; Tysnes et al. 1999). These molecules have extensively been demonstrated to enhance motility, proliferation, and survival, both in vitro and in vivo, suggesting that the presence of vasculature in and around tumors enhances tumor progression not only by providing key nutrients to the cells but also by providing structural and insoluble signaling to tumor cells (Demuth and Berens 2004; Kaufman et al. 2005; Kawataki et al. 2007; Lathia et al. 2012).

In addition to presenting biochemical cues that enhance GBM progression, the ECM also encodes structural and mechanical cues that can promote tumor progression. Cells mechanically engage their environment and respond to microenvironmental forces using a suite of molecular mechanisms whose actions are collectively known as mechanosensing and mechanotransduction (Discher et al. 2005). In order to probe the mechanical properties of its environment, a cell must actively apply a deformation force to the matrix. Canonically, cells generate force via contraction of the actin-myosin cytoskeleton, which is then transmitted to ECM proteins, such as fibronectin or collagen, through adhesive complexes containing integrins (e.g., focal adhesions) that physically connect the ECM to the cytoskeleton. These adhesive complexes then respond to stress/strain generated across the adhesive complex and activate downstream signaling pathways that result in changes of focal adhesion size and composition, force generation, and the regulation of gene transcription (Oakes et al. 2012; Kolega et al. 1991; Chang and Kumar 2013; Geiger and Bershadsky 2001; Solon et al. 2007; Pelham and Wang 1997; Maniotis et al. 1997).

Clinical observations and molecular profiling support the hypothesis that tissue stiffness and the corresponding mechanosensitive machinery are critical for the progression of a variety of solid tumors, including GBM. Ultrasound imaging of strain magnitudes during GBM resection suggests that the margins of GBM tumors are substantially stiffer than the adjacent normal tissue, although it is unclear whether the increase in stiffness is the result of a change in the abundance, type, or cross-linking of ECM components. Additionally, the direct mechanism through which this may contribute to tumor progression remains to be extensively detailed in GBM (Selbekk et al. 2010). Another intriguing clinical observation is that tumor cells often diffusely migrate along structures in the brain that are intrinsically stiff, such as the basolateral membrane of tumor-associated blood vessels and white matter tracts, known collectively as the structures of Scherer (Scherer 1938, 1940). This observation suggests that the mechanical environment may provide efficient "highways" that direct the cells to migrate away from the primary tumor.

GBM cells also display distinct molecular aberrations suggesting that they may co-opt and alter their mechanosensing machinery as a means of enhancing their malignancy. For example, a critical and nodal mechanosensitive protein, focal adhesion kinase (FAK), is often overexpressed and mutated in GBM, which results in enhanced signaling in many pro-growth pathways (Rutka et al. 1999). Aberrant signaling and regulation of many other mechanosensitive proteins has also been observed in GBM, further demonstrating how GBM cells co-opt normal mechanosensing processes (Belot et al. 2001; Friedlander et al. 1996; Paulus et al. 1993).

Given the extensive changes to the ECM, mechanosensitive machinery, and clinical observations, it is clear that the regulation of the ECM-cell interaction is substantially dysregulated in GBM and actively contributes to tumor progression. Therefore, the composition and mechanical properties of the ECM may represent an integrative target for effective therapeutics.

5.3 Engineering Advanced Models of the GBM Microenvironment

The past decade has seen a dramatic expansion of in vivo models of GBM, including genetically engineered mouse models (Huse and Holland 2009) and patient-derived xenograft models (Tentler et al. 2012), and these platforms have contributed to mechanistic studies of GBM progression as well as preclinical evaluation of therapies. However, the complexity, expense, and limited throughput associated with these models make them impractical as a first-line platform for discovery and screening. At the same time, it is widely acknowledged that the traditional in vitro approach of culturing cells on glass or plastic surfaces in high concentrations of soluble growth factors omits key elements of the tumor microenvironment. This gap has created a need for next-generation culture technologies that retain the scalability and parallelization of traditional culture approaches while incorporating the complexities of the in vivo microenvironment. Recognizing an opportunity to address these needs, investigators are increasingly leveraging the tools of biomaterials science and microfabrication to create these new culture platforms.

5.3.1 Traditional Culture Systems

Two-dimensional (2D) culture systems have historically been the workhorse of in vitro systems for reductionist cell biology, including the study of ECM regulation of GBM. In this context, 2D culture systems generally consist of glass or plastic surfaces coated with a thin layer of ECM protein, proteoglycan, or reconstituted matrix preparation (e.g., Matrigel; see discussion below) to support cell adhesion. These systems greatly facilitate optical imaging and the recovery of cellular material for downstream analysis (e.g., Western blot, RT-PCR, RNA-seq, etc.). These straightforward approaches have established a critical foundation for the field's understanding of many aspects of ECM regulation of GBM, including the roles of specific matrix components (fibronectin, vitronectin, and HA) and matrix metalloproteases (Giese et al. 1995; Koochekpour et al. 1995; Belien et al. 1999). However, the simplicity, ease of use, and experimental accessibility of these 2D cell culture systems also create a major drawback; it has become increasingly recognized that cells in three-dimensional (3D) systems behave quite differently than

cells in 2D culture (Griffith and Schwartz 2006; Yamada and Cukierman 2007). In fact, the differences in presentation, organization, and polarity of ECM proteins in a 3D matrix can cause cells to respond to ECM cues in ways that would be impossible to predict from their responses to the same proteins on 2D monolayers. This was first demonstrated in pioneering studies in which the behavior of malignant breast cancer cells could be normalized simply by placing them in a 3D microenvironment (Weaver et al. 1997). Additionally, the stiffness of conventional culture dishes is many orders of magnitude above what is typically experienced by cells in vivo, which can disrupt normal tissue morphogenesis and amplify pro-oncogenic signaling (Elkin et al. 2007; Paszek et al. 2005). To overcome this limitation, researchers have begun to exploit advances in polymer science and engineering to develop systems that enable precise control of the physical and chemical properties of the extracellular environment while maintaining ease of use for conventional biological analysis. These systems offer researchers unparalleled insight into the mechanisms underlying GBM-ECM interactions.

5.3.2 Polymer-Based 3D Matrices

The use of native, biologically derived hydrogels was the next iteration in developing highly controlled GBM ECM mimics. These biopolymers, including HA, collagen I, and Matrigel—a laminin-rich ECM extracted from mouse sarcomas—are derived from living sources, purified, and then reconstituted in vivo. These systems have been used to study the mechanics of GBM growth and invasion. For example, U87 MG glioma spheroids grown in 3D Matrigel plugs exert both traction forces on the gel as cells radially invade and compressive forces as the spheroid expands (Gordon et al. 2003). Further studies including expanded cell lines and GBM models and more expansive matrix choices for 3D culture showed that the 3D matrix often presents multiple levels of feedback controlling tumor growth and invasion. Yang et al. modified collagen network architecture by specifically controlling collagen gelation temperature, finding that pore size is a key determinant of glioma invasive speed (Yang et al. 2010). It is rather impractical to decouple many of these variables using reconstituted matrices; therefore intensive efforts have been directed to developing matrices that offer more flexibility, control, and experiment-to-experiment repeatability. The adaptation of synthetic polymers for use as cell culture models has provided impressive customization and repeatability for specific cell culture properties (as previously reviewed in Seliktar 2012; Lutolf and Hubbell 2005).

Using purely reconstituted matrices in the study of GBM is further complicated by the unique geometry of the brain GBM, which largely lacks the 3D fibrillar architecture observed in most other connective tissue ECMs (Thorne and Nicholson 2006). Our laboratory has taken an active interest in developing 3D matrices that mimic the unique architecture of the GBM-ECM by adopting an approach that

merges the use of biologically derived hydrogels and synthetic hydrogels. In this system, HA is functionalized with methacrylate groups, which can be covalently conjugated to macromolecules functionalized with thiol groups via Michael addition chemistry. In our initial study with this system, we functionalized the matrix with a cysteine-terminated peptide containing the cell-adhesive RGD sequence and then cross-linked the HA backbone with dithiothreitol, which contains two thiol groups. This dual functionalization approach offered us independent control of cell-adhesive ligand density and stiffness over more than three orders of magnitude (Ananthanarayanan et al. 2011). Importantly, this matrix exhibited small-pore structures devoid of fibrils, similar to the architecture of native brain tissue. In addition to replicating the structural environment of GBM, this system contains many chemical components of the pathological GBM environment, including the presence of both the CD44 and RGD ligands that arise in the tumor from the overproduction of HA and fibronectin, principally. Using this system, we found that increasing the stiffness of the 3D matrix inhibited tumor spheroid invasion into the surrounding matrix when cell attachment to the matrix is primarily through integrin-RGD interactions or HA-CD44 interactions, suggesting that this platform may hold potential as a reductionist in vitro system for studying mechanisms that underlie GBM-ECM interactions that lead to enhanced tumorigenesis (Kim and Kumar 2014).

5.3.3 Microfabricated Platforms

Researchers have begun to adopt and leverage techniques initially developed to help reduce the size of transistors, most notably soft lithography, microfluidics, and light-based patterning, to improve the spatial precision with which mechanical and biochemical cues can be presented to cells (Khademhosseini et al. 2006; Ross et al. 2012; Polacheck et al. 2013). While these approaches do not, in general, allow true 3D encapsulation of cells, they are massively parallelizable and enable the creation of microenvironments that can be engineered on the length scale of a single cell (10 nm–10 μm). One of the first studies to investigate how microtopography can influence GBM behavior was performed by Zhu and colleagues, who used laser irradiation to create periodic ridges spaced a few hundred nanometers apart and reported that C6 rat glioma cells aligned parallel to the direction of the grooves (Zhu et al. 2004). Subsequent studies suggested that cells not only align parallel to grooves in a surface but also migrate persistently parallel to the groove, a physical guidance mechanism that may partially explain GBM's preference to migrate along preformed structures in the brain architecture (Gallego-Perez et al. 2012). Our laboratory has expanded upon these studies by developing 3D confinement structures made of polyacrylamide that present cells with topographical guidance cues within a 3D environment. Using these microfabricated devices, we found that topographical migration, as observed in 2D systems, is present in 3D confinement channels and that narrow channels increase persistent migration regardless of the

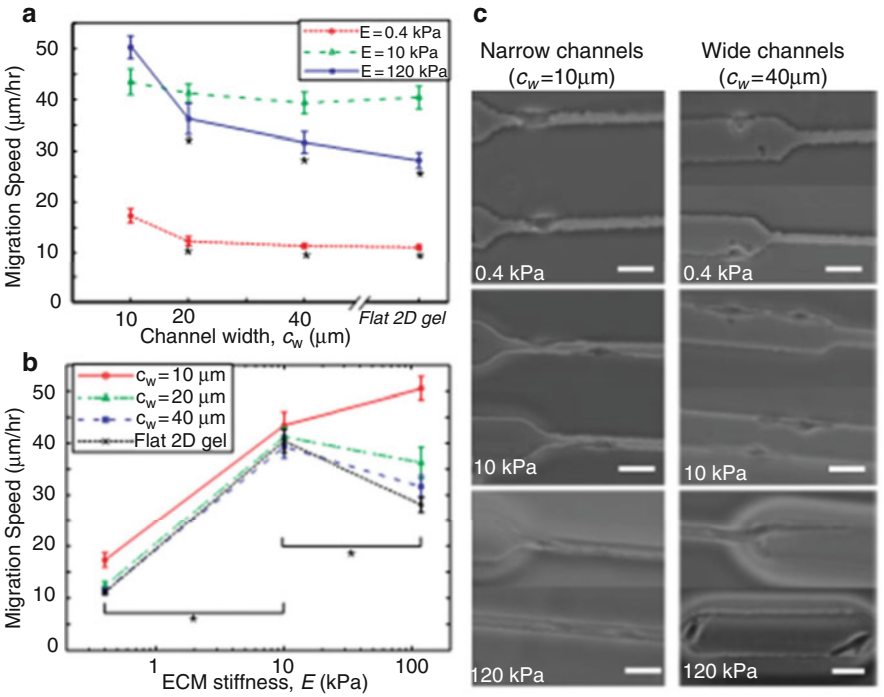

Fig. 5.1 Orthogonal regulation of GBM cell motility by ECM stiffness and topographical confinement. The migration of U373-MG cells was studied using polyacrylamide-based microfabricated channels coated with fibronectin. Channel width, c_w, and wall stiffness, E, were varied independently. Confinement in narrow channels increased migration speed for all values of wall stiffness (**a**) and relieved the inhibitory effect of high stiffness (**b**). Phase contrast images of the migrating cells are shown in (**c**). Figure adapted from Pathak and Kumar (2012), with permission

stiffness of the surrounding matrix (Fig. 5.1, Pathak and Kumar 2012). Our recent work studying the microchannel migration of breast cancer cells overexpressing the oncogene ErbB2 suggests that the physical microenvironment and oncogenic transformation can contribute to malignancy in an integrative manner (Pathak and Kumar 2013).

5.3.4 Molecular Discovery and Screening

While the sophistication of approaches for recapitulating and controlling the GBM microenvironment continues to improve rapidly, the field still lacks the ability to interrogate large parameter spaces, in which hundreds, or even thousands, of different parameter combinations can be tested systematically and simultaneously. This capability is critical because multiple parameters may interact in ways to

influence cell behavior that are impossible to predict from results of studies in which only one variable is modulated in isolation (Engler et al. 2004). Dissecting this nonlinear integration by cells of multiple simultaneous inputs represents a grand challenge in understanding ECM regulation of cancer biology.

The robotic spotting of ECM proteins has emerged as a highly useful, versatile, and high-throughput means to test the effects of ECM on cells. In this method, an array of microneedles is "inked" in an ECM protein and is robotically controlled to stamp the protein in a specific location on a target substrate. This process can be applied sequentially, and given the extremely precise control over the stamping location, different proteins, can be applied to thousands of independent spots (Fernandes et al. 2009). In this manner, an extremely high-throughput culture surface can be created that varies multiple parameters simultaneously. In one pilot study using this technology, Alberti et al. found that specific, but highly unpredictable, combinations of collagens I, II, and IV, laminin, and fibronectin controlled embryonic stem cell differentiation (Alberti et al. 2008). Similarly, ligand type and substrate stiffness can be varied using a robotic spotter attached to a UV light to initiate photopolymerization of a hydrogel precursor, with one study screening 17,000 distinct combinations of stiffness and ligand in one experiment (Anderson et al. 2004; Nakajima et al. 2007). Intriguingly, the photopolymerization used to create hydrogels in this experiment has micron-level resolution, allowing for the high-throughput investigation of mechanical inputs on a length scale smaller than that of a cell, an area of much needed investigation.

While these experiments offer unprecedented experimental power, they still require expensive and highly specialized instrumentation. As an alternative approach, we have developed a high-throughput system that can vary substrate stiffness and ligand orthogonally while maintaining ease of use and accessibility for investigators with standard biological and microfabrication capabilities. Our technique relies on the use of light to initiate orthogonal chemistries that can be leveraged to attach an ECM protein of choice and vary the mechanical properties of a gel (Rape et al. 2015). This system is based on HA, which has been modified to allow for specific light-based polymerization and functional group uncaging. Using the parallelization provided by the platform, we were able to condense experiments that would typically require hundreds of independent hydrogels onto a single substrate. We used this system to study the expression of the oncogenic microRNA, miR18a, in GBM cells and found that the microRNA is nonlinearly regulated by both stiffness and ECM in highly unpredictable ways (Fig. 5.2). While this pilot study was conducted as a 2D experiment, the platform could potentially be extended to more complex situations such as 3D geometries, as the specific chemistries used to perform the patterning are amenable to two-photon patterning, a highly precise means to locally control chemical processes in 3D matrices.

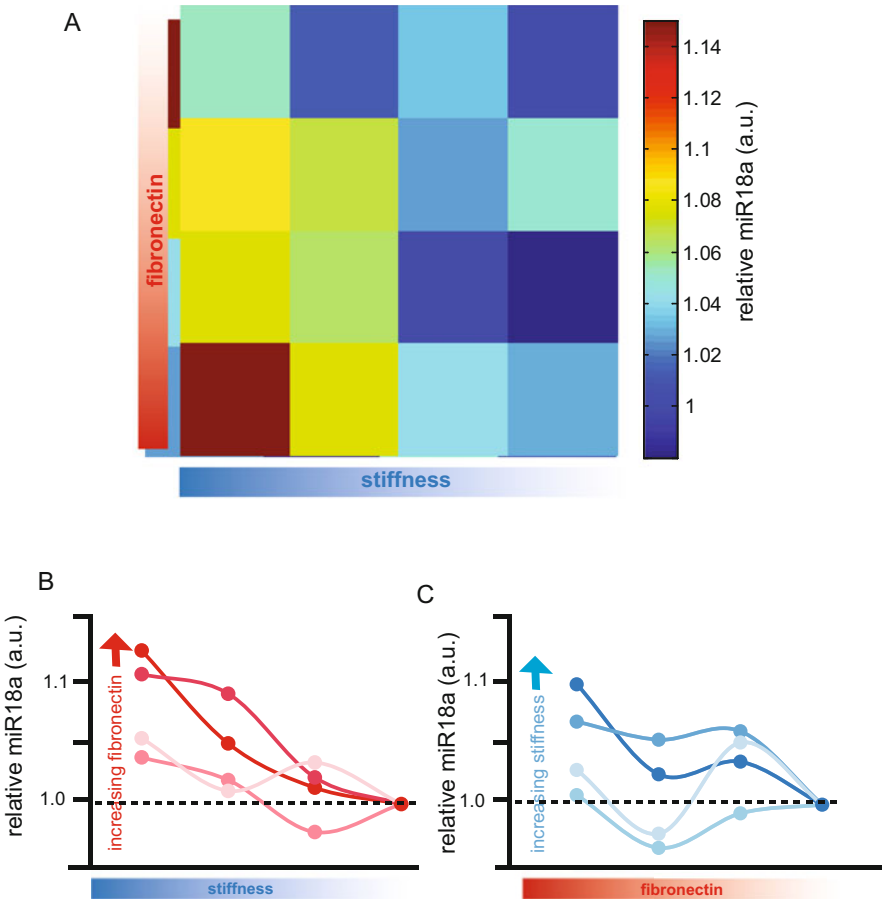

Fig. 5.2 ECM-sensitive regulation of mir18a using dual gradient-patterned hydrogels. U373-MG cells were incubated with probes for mir18a for 16 h on HA-based hydrogels, and mir18a expression was quantified based on the fluorescent signals of individual cells. Mir18a expression was quantified for 16 unique matrix stiffness ligand combinations (**a**). Iso-fibronectin curves (*light red* to *dark red* equals low fibronectin to high fibronectin) show that substrate stiffness regulated miR18a expression at all fibronectin densities tested (**b**). Similarly, iso-stiffness curves (*light blue* to *dark blue* equals low stiffness to high stiffness) show that fibronectin density only regulates miR18a expression at high stiffness (**c**). Figure adapted from Rape et al. (2015), with permission

5.4 Future Directions

The use of engineered microenvironments is catalyzing a revolution in the understanding of how GBM cells interact with their environment and how these interactions lead to malignancy. This profound innovation, however, is only the beginning of a trajectory that promises to simplify, expedite, and increase the efficiency of the drug discovery process. Such efforts range from the identification of new

therapeutic targets to streamlining the drug development pipeline, analogous to the current development organ-on-a-chip platforms for drug screening in other human tissues, including the heart and lungs (Huh et al. 2010; Mathur et al. 2015). To achieve this broad vision, a number of key barriers must be overcome.

First, the ability to simultaneously control multiple environmental parameters within a single, unified 3D space needs to be developed. Our own work illustrates the utility of photochemistry to create microenvironments with defined physical properties. Other labs are developing more sophisticated and customizable light-based chemistries, including the adoption of "click" chemistry for biological applications (DeForest et al. 2009). A next challenge will be to pattern multiple cell types at specific positions within these devices. Recent work has suggested that affixing DNA-based affinity tags to the cell surface may provide a useful way to organize large numbers of cells in a highly precise manner through DNA hybridization (Todhunter et al. 2015). The use of 3D printing to deposit cells selectively in three dimensions also holds great promise for this purpose (Murphy and Atala 2014). Perhaps the final hurdle to clear in developing a fully functional GBM mimic is vascularization of the tissue, which has historically been a challenge in tissue engineering due to difficulties in recapitulating complex cellular architectures (e.g., endothelial cells vs. pericytes vs. smooth muscle cells), delivering oxygen and nutrients, and integrating vasculature over culture-wide length scales. In GBM, the challenge is compounded by the fact that tumor vasculature is often structurally and functionally aberrant relative to host vasculature. New cell- and matrix-patterning technologies, when paired together with strategies to release provascular growth factors, should help accelerate progress in this area (Moon and West 2008).

Once developed sufficiently, GBM-mimetic culture systems could be combined with other reverse-engineered systems of the tumor microenvironment, perhaps leading to a sort of "tumor on a chip." The successful development of these and related technologies may immensely increase the speed and efficiency of the drug discovery process by bridging the gap between the culture plate and the animal model.

5.5 Conclusions

The physical components of the ECM microenvironment of GBM make up a complex system that contains many signals that contribute to tumor promotion and malignancy. Using the tools of chemistry, materials science, and microfabrication, researchers have developed sophisticated reductionist platforms that enable the direct interrogation of the biophysical interactions that lead to GBM progression. The further development of a truly mimetic tumor microenvironment will lead to not only a greater understanding of disease mechanisms but also yield platforms that facilitate high-throughput drug discovery and validation.

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Chapter 6
Breaching and Opening Basement Membrane Barriers: The Anchor Cell Leads the Way

Daniel Keeley and David R. Sherwood

Abstract Metastasis is initiated in epithelial-derived tumors when cells at the tumor front breach the epithelial basement membrane (BM). Invasion through BMs is thought to be one of the most rate-limiting steps in cancer progression and thus is a therapeutically attractive target for halting tumor spread. Despite intense interest, it has been challenging to experimentally determine how invasive cells breach and clear BM barriers, which has hindered efforts to block metastasis. Here we discuss how an experimentally tractable developmental invasion event, anchor cell (AC) invasion in the model system *C. elegans*, is offering powerful new insights into the fundamental mechanisms that invasive cells use to breach BM barriers and how cells at the breach site widen BM gaps through a new mechanism called BM sliding. Finally, we cover studies demonstrating that AC invasion can also be used as a new paradigm to examine how alterations in the tumor microenvironment impinge on cell invasive behavior.

6.1 Introduction

Basement membranes (BMs) are thin, dense, sheet-like forms of extracellular matrix that underlie all epithelia and surround blood vessels, muscles, adipocytes, and Schwann cells (Halfter et al. 2015; Yurchenco 2011). Two polymeric protein networks shape BM structure: a cell-associated assembly of laminin molecules and a polymer of type IV collagen proteins. The independent laminin and type IV collagen networks are thought to be connected through other BM proteins, including the glycoprotein nidogen and the heparan sulfate proteoglycans perlecan and agrin (Behrens et al. 2012; Fox et al. 1991; Hohenester and Yurchenco 2013). BMs provide tissues with mechanical and barrier support and harbor growth factors, differentiation signals, and polarity cues (Halfter et al. 2015; Poschl et al. 2004; Yurchenco 2011). The type IV collagen network of BMs is covalently cross-linked through multiple distinct bonds, which helps impart the mechanical and barrier properties to BMs (Fidler et al. 2014; Khoshnoodi et al. 2008; Vanacore et al.

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2009). Transmission electron microscopy studies have indicated that most BMs are approximately 50–100 nm thick; however, more recent atomic force microscopy measurements that allow for the retention of water have revealed that BMs are twofold thicker (Candiello et al. 2007; Halfter et al. 2015). The genes that encode BM proteins are ancient and emerged concurrently with animal multicellularity (Hynes 2012; Ozbek et al. 2010), suggesting that BMs were required to construct and organize tissues. An important implication from its early origins is that the mechanisms that cells use to build, maintain, and remodel BMs are likely evolutionarily conserved between animals (Sherwood 2015).

The network of laminin, type IV collagen, and associated glycoproteins that make up BMs creates a dense meshwork with a pore size ranging from roughly 10 to 130 nm, a diameter far too small for a cell to traverse without removing this barrier (Abrams et al. 2000, 2003; Rowe and Weiss 2008; Wolf et al. 2013; Yurchenco and Ruben 1987). Yet cells repeatedly cross through BMs to enter new tissues during development and normal physiological functions in a process called cell invasion or BM transmigration. For example, BM barriers are crossed during the numerous epithelial-to-mesenchymal transitions that allow cell dispersal and organ formation in development (Cheung et al. 2005; Kelley et al. 2014; Nakaya et al. 2008; Yang and Weinberg 2008). BMs are also traversed during leukocyte trafficking across vascular BMs, and endothelial cells cross the vascular BM to form new blood vessels (Seano et al. 2014). The presence of BMs thus creates a paradox in that they are crucial in providing tissue support and barrier functions; however, they must be repeatedly removed and traversed by cells during development and normal organ function. A solution to this problem has been solved in vascular BMs by the formation of specialized regions with preformed openings as well as reduced BM regions that allow leukocyte trafficking (Baluk et al. 2007; Pflicke and Sixt 2009; Voisin et al. 2009, 2010; Wang et al. 2006). In most cases of BM crossing, however, de novo BM openings must be created to facilitate BM transmigration (Kelley et al. 2014). Epithelial-derived cancers, which account for 90% of cancer-related deaths, also make openings in BMs during tumor progression (Frei 1962; Rowe and Weiss 2008). BM breaching initiates metastasis and is associated with poor patient prognosis (Barsky et al. 1983; Hagedorn and Sherwood 2011). BM invasion is thought to be one of the most rate-limiting aspects of metastasis and an attractive therapeutic target in cancer (Christofori 2006; Madsen and Sahai 2010; Nguyen et al. 2009; Steeg 2003). Thus, understanding how cells transmigrate this barrier is of great interest in order to develop new treatment strategies to curb metastasis.

Cell invasion events are often stochastic and occur deep in tissues in vertebrates. As a result, cell invasive behavior is difficult to visualize with microscopy approaches (Beerling et al. 2011). Furthermore, revealing mechanisms underlying invasion require genetic manipulation of the invading cell, the BM, and the tissue the cell is invading—a prospect that is currently prohibitively time-consuming and expensive in vertebrates. Thus, progress in defining the cellular and molecular regulators in BM cell invasion has been made largely in in vitro and sophisticated ex vivo assays using reconstituted matrices or isolated BMs (Rowe and Weiss 2008;

Schoumacher et al. 2010, 2013). These studies have identified key invasive cellular structures and molecular components associated with invasion; however, these assays do not faithfully recapitulate the in vivo cellular environment nor mimic the physiological relevant composition, cross-linking, or stiffness of cell-associated BMs (Even-Ram and Yamada 2005; Lokman et al. 2012; Rowe and Weiss 2008; Schoumacher et al. 2010, 2013; Wu et al. 2012). Thus, our knowledge of the mechanisms that facilitate the breaching and removal of BM barriers is incomplete.

The anchor cell (AC) is a specialized uterine cell in *Caenorhabditis elegans* that invades through the BM separating the uterine and vulval tissue in a highly stereotyped manner during a 90-min window (Sherwood and Sternberg 2003). AC invasion initiates the attachment of the uterine and vulval tissue during development—a connection necessary for mating and egg-laying in the adult worm. AC invasion is the first in vivo animal model that combines genetic analysis with live-cell subcellular resolution of cell-BM interactions during invasion and BM remodeling (Fig. 6.1; Hagedorn and Sherwood 2011). Many features make this an outstanding model for examining cell invasion. The predictability of this invasion event and its amenability to rapid screening make it ideal for identifying genes that control invasion (Hagedorn et al. 2009; Matus et al. 2010; Schindler and Sherwood 2011; Wang et al. 2014a; Ziel et al. 2009a, b). The structure of the *C. elegans* BM is conserved with that of vertebrates, and most components—including the major structural components laminin and type IV collagen—have been functionally tagged with GFP and GFP derivatives to allow a dynamic visualization of invasion (Fidler et al. 2014; Fitzgerald and Schwarzbauer 1998; Hagedorn et al. 2009; Hesselson et al. 2004; Kramer 2005). Further, CRISPR-mediated tagging, optical highlighting, and photo-bleaching methods allow BM components, AC-expressed proteins, and cell membrane components to be followed dynamically during invasion (Hagedorn et al. 2014; Ihara et al. 2011; Matus et al. 2014; Morrissey et al. 2014). In addition, cell-specific RNAi, cell-specific protein degradation, and temporally controlled cell-specific expression techniques help determine the underlying roles of proteins and genes in invasion and BM remodeling (Armenti et al. 2014; Hagedorn et al. 2009). Finally, methods for quantitative 4D live-cell imaging of cell-BM interactions have been developed to elucidate the cellular and subcellular mechanisms that drive BM transmigration (Hagedorn et al. 2013; Wang et al. 2014b).

This chapter focuses on AC invasion and BM hole widening after invasion during uterine-vulval attachment in the worm and how these findings have advanced our fundamental understanding of similar events in cancer. First we will review the mechanisms that regulate AC invasion, covering the transcriptional programs that prime the AC to invade and the dynamics of invasive structures that breach and clear BM barriers. Then we will discuss how the gap in the BM is widened after AC invasion to allow additional uterine and vulval cells to directly attach through a newly characterized mechanism called BM sliding. Finally, we will analyze AC invasion as a model for examining how extrinsic molecules in the microenvironment impact cell invasion, focusing on the matricellular protein

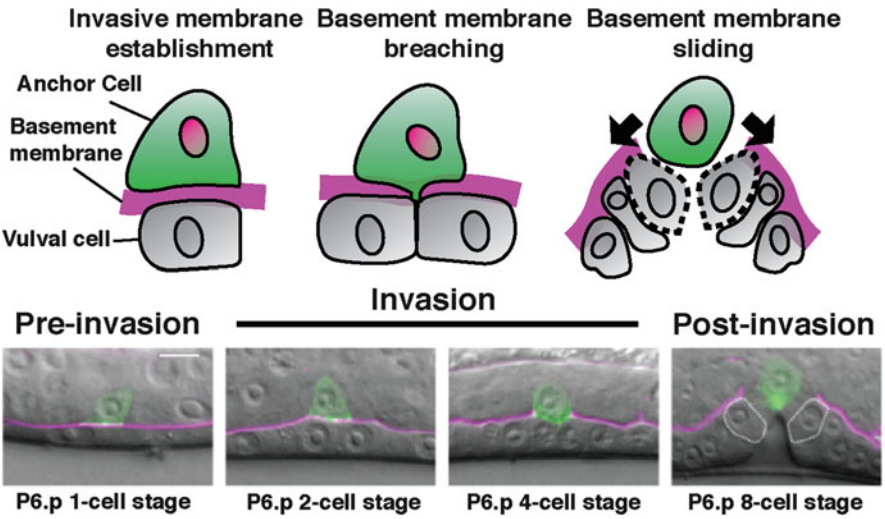

Fig. 6.1 Anchor cell (AC) invasion and basement membrane (BM) remodeling. *Top panel*: A schematic diagram highlighting the major developmental steps of AC invasion and BM remodeling during uterine-vulval attachment in *Caenorhabditis elegans* from *left* to *right*. The *dashed outline* on the *right panel* (and *panel below*) indicates the vulD cell, where the BM stops sliding. *Bottom panel*: Micrographs of AC invasion in vivo with the AC expressing a fluorescent reporter in *green* and the BM labeled with a fluorescent reporter shown in *magenta*. Invasion progresses from *left* to *right* with BM breaching occurring in the *middle panels*. AC invasion occurs in tight synchrony with the division of the underlying P6.p vulval cell and can be staged by the number of P6.p descendants (i.e., P6.p 1-cell stage, 2-cell, 4-cell, and 8-cell stages). *Scale bar* 5 μm (Image reproduced from Hagedorn and Sherwood (2011) with permission from Elsevier)

SPARC, which is overexpressed in many metastatic cancers and promotes invasive behavior.

6.2 Breaching the BM De Novo: AC Invasion

6.2.1 Transcription Factors Promoting Invasion

Cell invasion is a highly specialized process and depends on extensive changes in gene expression, such as the upregulation of actin regulators and matrix metalloproteinases, i.e., MMPs (Kelley et al. 2014; Murphy and Courtneidge 2011; Page-McCaw et al. 2007; Wang et al. 2004). The transcriptional networks that endow cells with the ability to breach BMs, however, are largely unclear. Identifying the regulatory networks that program invasiveness is crucial, as they may be the most promising therapeutic targets to broadly inhibit invasion (Ell and Kang 2013). The stereotyped nature of AC invasion and the ability to conduct large-

scale screens and characterize genes are allowing researchers to elucidate the transcriptional mechanisms that program invasiveness in cells.

The AC is first specified during the late L2 larval stage through a LIN-12/LAG-2 (Notch/Delta)-mediated lateral signaling interaction with a neighboring ventral uterine (VU) cell. In this interaction the AC upregulates the Notch ligand gene *lag-2* and is specified by the absence of Notch activation (Greenwald 2005). After the AC/VU fate decision, the AC expresses specific genes that are involved in invasion, including the protocadherin *cdh-3*, the integrin *pat-3*, and the RhoG GTPase *mig-2* (Hagedorn et al. 2009; Sherwood and Sternberg 2003; Ziel et al. 2009a). Several transcription factors, including the helix-loop-helix factor HLH-2 (vertebrate E protein) and the nuclear hormone receptor NHR-67 (vertebrate TLX), play roles in pro-AC competency, AC/VU Notch-mediated interactions (HLH-2 and NHR-67), and later pro-invasive differentiation (Karp and Greenwald 2004; Schindler and Sherwood 2011; Verghese et al. 2011). These transcription factors are expressed in the AC throughout its development and may belong to a dynamic transcriptional network that controls distinct transcriptional targets at different stages that direct both early specification and later pro-invasive differentiation of the AC (Schindler and Sherwood 2011).

Shortly after the AC's initial specification, the *nhr-67* (TLX) gene becomes upregulated and induces G1 cell-cycle arrest in the AC (Fig. 6.2; Matus et al. 2015). G1 cell-cycle arrest in development is strongly associated with cellular differentiation programs (Gonzales et al. 2015; Mummery et al. 1987; Ruijtenberg and van den Heuvel 2015). Strikingly, G1 cell-cycle arrest is similarly required for the AC to adopt features of an invasive cell (Matus et al. 2015). These characteristics include the expression of pro-invasive genes, such as the *C. elegans* MMPs *zmp-1, zmp-3*, and *zmp-6* and actin regulators, including the formin *exc-6* and the Ena/VASP ortholog *unc*-34 (Matus et al. 2015). G1 arrest is also required for the formation of invadopodia-specialized F-actin-rich subcellular protrusions that breach BMs. Consistent with this being a general feature of invasive cells, invasive ability is correlated with decreased cell proliferation in many cancer cell lines, tumor models, and human cancers, as well as with developmental invasion events (Gil-Henn et al. 2013; Hoek et al. 2008; Vega et al. 2004; Wang et al. 2004; Yano et al. 2014). As most chemotherapies target dividing cells (Yano et al. 2014), an important implication of these findings is that invasive cells may be more resistant to antiproliferative chemotherapy, leaving these cells unaffected and able to reenter the cell cycle at a later time.

The molecular mechanisms that connect G1 cell-cycle arrest with cellular differentiation are thought to involve cell-cycle-dependent alterations in chromatin that may permit the transcriptional activation of differentiation genes (Ma et al. 2015). Consistent with this idea, the conserved histone deacetylase, *hda-1*, a chromatin remodeling protein, is upregulated in the AC after G1 arrest and promotes pro-invasive gene expression and invadopodia formation (Matus et al. 2015). The zinc finger protein MEP-1, a component of the nucleosome remodeling NuRD complex that requires HDAC activity for its function, is also a crucial regulator of AC pro-invasive differentiation and might also be activated by G1 arrest (Leight

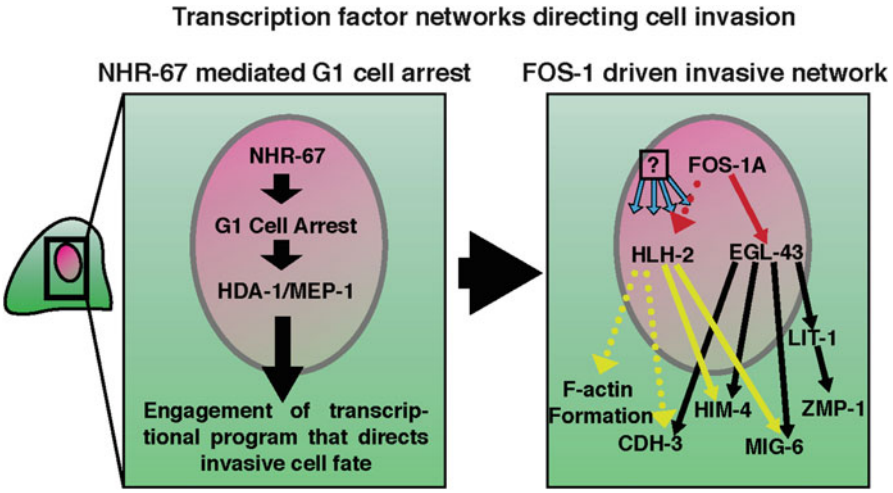

Fig. 6.2 Transcriptional networks programming AC invasiveness. *Left*: After AC specification, the transcription factor (TF) NHR-67 (vertebrate TLX) induces G1 arrest in the AC, which leads to chromatin remodeling through HDA-1 (HDAC) and MEP-1(NuRD complex) that allows pro-invasive gene expression and invasive cell fate differentiation. *Right*: G1 cell-cycle arrest promotes FOS-1A (c-Fos proto-oncogene) expression, which regulates the expression of other TFs such as HLH-2 (E protein) and EGL-43 (EVI1), which in turn direct the expression of a number of pro-invasive genes encoding the proteins CDH-3 (protocadherin), HIM-4 (hemicentin), MIG-6 (papilin), and ZMP-1 (MMP), as well as proteins that generate F-actin. The *solid arrows* indicate strong regulation, and *dashed arrows* indicate partial regulation. The "*question mark*" represents TFs that remain to be identified, as FOS-1A is not required for the expression of many pro-invasive genes in the AC

et al. 2015; Matus et al. 2010; Zhang et al. 1998). After the AC has entered G1 cell-cycle arrest, a transcription factor network involving the bZIP transcription factor FOS-1A, HLH-2 (vertebrate E proteins), and the zinc finger protein EGL-43 (an ortholog of the vertebrate oncogene EVI1) directs pro-invasive gene expression (Fig. 6.2; Rimann and Hajnal 2007; Schindler and Sherwood 2011; Sherwood et al. 2005). How G1 cell-cycle arrest activates this network is unclear but may involve transcriptional upregulation, as HDA-1 and MEP-1 promote *fos-1a* gene upregulation in the AC (Matus et al. 2010). Within this network, the FOS-1A protein regulates *egl-43* (EVI1) expression and promotes increased expression of the *hlh-2* gene (Rimann and Hajnal 2007; Schindler and Sherwood 2011). The HLH-2 protein is required for the expression of several downstream invasion effector genes, including those encoding the extracellular matrix proteins MIG-6 (papilin) and hemicentin. HLH-2 also increases the expression of the *cdh-3* (protocadherin) gene (Schindler and Sherwood 2011). In addition, the loss of HLH-2 disrupts actin formation at the invasive membrane of the AC, which is necessary for invasion to occur (Schindler and Sherwood 2011). FOS-1A, either directly or indirectly, is required for AC expression of the MMP *zmp-1*, the matrix protein hemicentin, and increased *cdh-3* expression (Hwang et al. 2007; Sherwood

et al. 2005). Interestingly, EGL-43 negatively regulates expression of the gene encoding the adhesion protein MIG-10 (lamellipodin), which is a positive target of FOS-1A (Wang et al. 2014a, c). This may establish a system where a circuit of transcription factors maintain basal levels of MIG-10 protein, as over- or underexpression of the *mig-10* gene negatively impacts invasion. EGL-43 also drives *lit-1* (Nemo-like kinase), which is upstream of *zmp-1* expression (Matus et al. 2010).

The vertebrate orthologs of the FOS-1A, EGL-43, and HLH-2 proteins are strongly associated with promoting invasion in normal development and in numerous epithelial-derived cancers (reviewed in Ozanne et al. 2006; Rimann and Hajnal 2007; Sherwood et al. 2005; Young and Colburn 2006), suggesting this regulatory network is conserved. Other transcription factors will certainly be added to the AC invasive fate network, as many AC-enriched genes that promote invasion, such as *cdc-37* (Hsp90 co-chaperone), *mig-2* (RhoG), and *pat-3* (integrin), are not regulated by these transcription factors (Matus et al. 2010; Schindler and Sherwood 2011; Shekarabi et al. 2005).

6.2.2 *Establishing the Invasive Cell Membrane*

Downstream of transcriptional changes, the AC establishes a unique plasma membrane domain at the cell-BM interface termed the invasive cell membrane (Fig. 6.3a). The heterodimeric transmembrane integrin receptor αINA-1/βPAT-3 and the transmembrane UNC-6 (netrin) receptor UNC-40 (vertebrate DCC) play important roles in invasive membrane establishment and BM adhesion (Hagedorn et al. 2009, 2013; Wang et al. 2014b; Ziel et al. 2009a; Ziel and Sherwood 2010). The *C. elegans* αINA-1/βPAT-3 integrin is most similar to vertebrate BM-laminin-binding integrins (Baum and Garriga 1997). INA-1/PAT-3 expression is upregulated in the AC at the time of AC specification, and the INA-1/PAT-3 heterodimer localizes to the invasive cell membrane. The targeted loss of integrin activity in the AC, however, only slightly reduces AC-BM attachment (Hagedorn et al. 2009). UNC-40 (DCC) may also help adhere the AC to the BM, as loss of UNC-40 or its ligand UNC-6 (netrin) results in weak AC-BM adhesion defects (Ziel et al. 2009a). MIG-10, an ortholog of mammalian lamellipodin, also participates in AC-BM adhesion although its precise role is unclear (Wang et al. 2014c). As the BM adhesion receptor dystroglycan is also expressed in the AC, a number of BM adhesion receptors may play redundant roles contributing to AC-BM attachment (Johnson et al. 2006).

While INA-1/PAT-3 (integrin) plays a minor role in AC-BM adhesion, it is crucial in establishing the invasive cell membrane and promoting cell invasion. The invasive membrane is enriched in actin regulators, F-actin, and the phosphoinositide PI(4,5)P_2 and is the site of polarized secretion and active membrane trafficking (Fig. 6.3a; Hagedorn et al. 2009, 2014; Ziel et al. 2009a). In response to ligand binding, integrins become activated then cluster and recruit

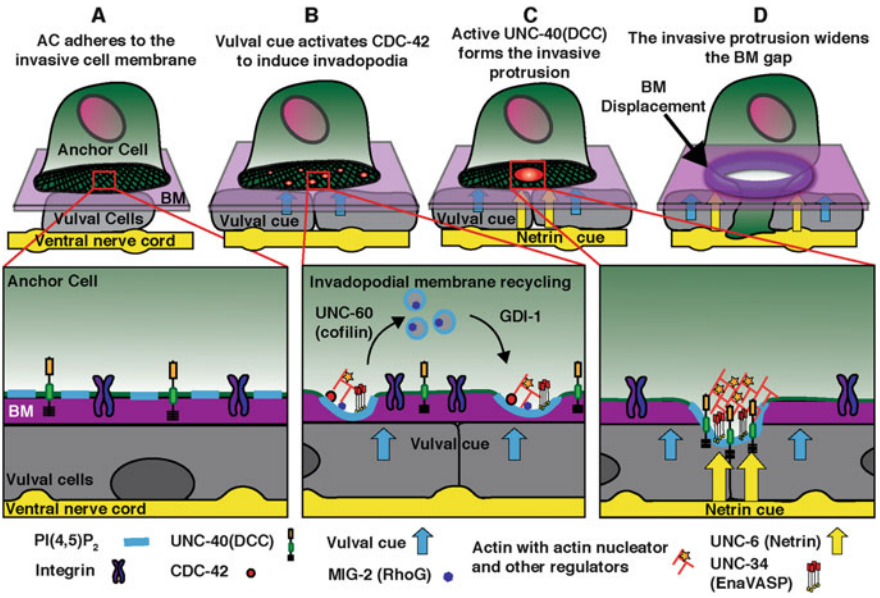

Fig. 6.3 AC breaching and clearing the BM. (**a**) The AC adheres to the BM with integrin, UNC-40 (DCC), and likely other adhesion receptors and establishes the invasive cell membrane—a membrane domain that is rich in F-actin and actin regulators, signaling molecules, vesicle trafficking, and invadopodia. (**b**) Just prior to invasion, the vulval cells secrete an unidentified diffusible "vulval cue" that activates CDC-42 and promotes the seeding of robust F-actin-rich invadopodia that breach the BM. Invadopodia are formed with an F-actin core and a specialized invadopodial membrane that is actively recycled through the endolysosome. Recycling is mediated by UNC-60A (cofilin) and GDI-1 (Rab GDP disassociation inhibitor). UNC-60A (cofilin) also disassembles F-actin at invadopodia to promote rapid invadopodia turnover. (**c**) The UNC-40 (DCC) receptor traffics to the BM breach site, where in response to UNC-6 (netrin) secreted from the ventral nerve cord is activated and recruits its effectors Ena/VASP and MIG-2 (RhoG) to build the invasive protrusion. (**d**) The invasive protrusion displaces the BM physically, and BM proteins accumulate at the edges of the BM opening

adaptor and signaling proteins that mediate F-actin formation and membrane trafficking to the cell surface (Cluzel et al. 2005; Wickstrom and Fassler 2011). Consistent with a key signaling function for integrin at the invasive membrane, a loss of INA-1/PAT-3 in the AC strongly disrupts the recruitment of all known F-actin regulators that localize to this domain and reduces or eliminates F-actin formation. Integrin is also required for the localized BM accumulation of hemicentin, an extracellular matrix protein that promotes invasion (Hagedorn et al. 2009; Morrissey et al. 2014). UNC-40(DCC) also plays a role in establishing the invasive cell membrane. UNC-40 is polarized to the invasive cell membrane by its ligand UNC-6 (netrin), which is secreted from the ventral nerve cord and accumulates in the BM under the AC (Ziel et al. 2009a). UNC-40(DCC) recruits its effectors MIG-2 (RhoG), CED-10 (Rac), UNC-115 (an actin regulator), and

UNC-34 (Ena/VASP), which build the invasive protrusion that crosses the BM (Wang et al. 2014a).

6.2.3 *AC Invadopodia and the Invasive Protrusion: Breaching and Clearing the BM*

The basally localized invasive cell membrane is an F-actin-rich domain that supports polarized secretion, BM adhesion, and invadopodia formation. Invadopodia are F-actin-rich membrane protrusions that form at sites of matrix removal and have been hypothesized to be the cellular drill bits of invasive cells, mediating invasion through BM barriers (Lohmer et al. 2014). Though studied in vitro for over 30 years, invadopodia function and even existence in vivo have been difficult to establish because visualizing cell invasion in native tissue environments remains challenging (Chen 1989; David-Pfeuty and Singer 1980; Linder et al. 2011; Murphy and Courtneidge 2011).

The development of 4D live-cell imaging approaches during AC invasion revealed that at least 3 h prior to invasion, numerous small (~1.0 μm), protrusive, F-actin-rich invadopodia form and turn over rapidly (~45 s lifetimes) along the AC's invasive cell membrane (Hagedorn et al. 2013). Similar to invadopodia in cancer cell lines observed in vitro, AC invadopodia are dependent on integrin activity and contain numerous actin regulators, including the ADF/cofilin ortholog UNC-60A, the Rho GTPases CDC-42 and MIG-2, and the Rac GTPase CED-10, the N-WASP ortholog WSP-1, and the Ena/VASP ortholog UNC-34 (Fig. 6.3b; Destaing et al. 2011; Hagedorn et al. 2013, 2014; Lohmer et al. 2016). CDC-42 and WSP-1 seed new invadopodia, and UNC-60A (cofilin) disassembles these structures. The function of other actin regulators in AC invadopodia is not yet clear, but the role of such regulators in the invadopodia of cancer cell lines has been studied in vitro (Bergman et al. 2014; Moshfegh et al. 2014).

The activity of these invadopodia in the AC appears tightly regulated. While hundreds of invadopodia form and turn over hours before BM breaching, usually only one or two invadopodia penetrate the BM during a highly stereotyped ~20-min period in the early-to-mid L3 larval stage (Hagedorn et al. 2013). The timing of invadopodia BM breaching is controlled by a diffusible cue from underlying vulval cells that activates CDC-42 in the AC. Activated CDC-42 is found at the invadopodium that breaches the BM and may promote more robust and protrusive invadopodia through WSP-1-directed F-actin generation and perhaps the recruitment of additional components such as proteases that "arm" invadopodia for BM penetration (Lohmer et al. 2016; Poincloux et al. 2009).

In addition to confirming the existence and regulation of invadopodia in vivo, studies in the AC have also revealed new key molecular and structural features of invadopodia. One of the most notable discoveries is that of a unique, actively recycled invadopodial membrane. During invadopodia formation, a specialized

membrane containing the phospholipid PI(4,5)P_2 and membrane-associated Rac and RhoG GTPases is rapidly recycled through the endolysosome during invadopodia assembly and disassembly (Fig. 6.3b; Hagedorn et al. 2014). Through genetic screens, several regulators of invadopodial membrane trafficking have been identified, including UNC-60A (cofilin) and GDI-1 (Rab GDP dissociation factor). UNC-60A appears to regulate the exocytosis of the invadopodial membrane at sites of nascent invadopodia and may promote trafficking through the endolysosomal system (Hagedorn et al. 2014). In the absence of UNC-60A, the invadopodial membrane is trapped in static internal vesicles within the AC and fails to traffic to invadopodia at the invasive cell membrane. GDI-1 is crucial for targeting the invadopodial membrane selectively to the basal invasive membrane. The loss of GDI-1 results in the invadopodial membrane being inappropriately trafficked to apical and lateral plasma membrane domains in the cell (Fig. 6.3b; Hagedorn et al. 2014; Lohmer et al. 2016). Although more difficult to examine because of the slower dynamics of invadopodial turnover in cell culture, similar recycling occurs in several cancer cell lines, suggesting that the invadopodial membrane is likely a shared aspect of invadopodia construction (Artym et al. 2011; Monteiro et al. 2013; Poincloux et al. 2009; Williams and Coppolino 2011). The invadopodial membrane may be required for the concentrated delivery of membrane-associated proteases and to provide a source of membrane addition for protrusive activity (Frittoli et al. 2014; Poincloux et al. 2009; Trimble and Grinstein 2015). The unique composition of the invadopodial membrane might also serve as an organizing platform for the recruitment of actin regulators, adhesion, and signaling proteins that direct invadopodia construction (Moshfegh et al. 2014; Yamaguchi and Oikawa 2010).

During BM breaching by an invadopodium, the netrin receptor UNC-40 (DCC) is trafficked to the breach site, although the mechanism of recruitment remains elusive (Morrissey et al. 2013). UNC-40 (DCC) at the BM breach is activated by its ligand UNC-6 (netrin) and, in response to activation, recruits effectors such as UNC-34 (Ena/VASP) and MIG-2 (RhoG) that promote focused F-actin formation to build a single large invasive protrusion that clears an opening in the BM (Fig. 6.3c, d; Hagedorn et al. 2013). In the absence of UNC-6 (netrin) or UNC-40 (DCC), a large protrusion never forms, and the clearing of an opening in the BM necessary for invasion is perturbed. Type IV collagen degradation products and proteases are expressed near sites of invasion in vivo and in vitro (Page-McCaw et al. 2007; Rowe and Weiss 2008). Thus, it has been generally assumed that BM is degraded and dissolved during invasion, which has led to extensive clinical trials to target MMPs in metastatic cancers. These clinical trials, however, were universally unsuccessful for reasons that remain unknown (Dufour and Overall 2013; Overall and Kleifeld 2006). Optical highlighting experiments using a photo-convertible form of laminin and type IV collagen in *C. elegans* have surprisingly revealed that the BM breached by the AC is physically displaced by the invasive protrusion (Fig. 6.3d; Hagedorn et al. 2013). While these results do not rule out a role for proteases in weakening BM, they indicate that invasion requires physical forces to clear a path through this barrier. This may help explain why clinical trials targeting

MMPs have been ineffective—invasive cells may invade independent of BM proteolysis.

The BM invasion program in the AC is likely conserved, as invadopodia have been observed in numerous invasive cancer cell types (Hoshino et al. 2013) and in invasive vertebrate embryonic and endothelial cells (Patel et al. 2012; Seano et al. 2014). Further, single invasive protrusions have been seen when tumor cells transmigrate BMs in ex vivo BM invasion assays (Hotary et al. 2006; Leong et al. 2014; Schoumacher et al. 2010). Notably, the *netrin-1* ligand is highly expressed and associated with invasion in the most aggressive metastatic cancers, including medulloblastoma, glioblastoma, malignant melanoma, and pancreatic adenocarcinoma (Akino et al. 2014; Dumartin et al. 2010; Kaufmann et al. 2009; Shimizu et al. 2012). Thus, the netrin pathway may guide invasive cells across BM barriers in highly metastatic cancers and be a particularly attractive target for cancer therapeutics.

6.3 Post AC Invasion: BM Sliding Further Opens the Breach

Shortly after the AC completes invasion, the opening in the BM enlarges beyond the boundaries of the AC (Fig. 6.4). This expansion of the BM gap allows additional uterine and vulval cells to directly attach and form the mature uterine-vulval connection (Schindler and Sherwood 2013). Large openings in BMs occur frequently in development, such as during vertebrate and invertebrate gastrulation, mouse distal visceral endoderm formation, and *Drosophila* imaginal disk eversion (Hiramatsu et al. 2013; Nakaya et al. 2008; Saunders and McClay 2014; Srivastava et al. 2007). Large gaps in BMs have been known for decades to occur in cancer at the tumor front, and these openings are thought to be crucial in allowing cancer cells to metastasize (Barsky et al. 1983; Frei 1962; Gabbert et al. 1985; Kobayashi et al. 1995; Rowe and Weiss 2008). It has been suggested that these large BM openings are created by proteolytic degradation based on type IV collagen degradation and protease expression (Hotary et al. 2006; Page-McCaw et al. 2007; Rowe and Weiss 2008; Sameni et al. 2009). Reduced BM synthesis and alterations in composition have also been proposed as mechanisms that may result in the loss of BM (Flug and Kopf-Maier 1995; Frei 1962; Spaderna et al. 2006).

Although it has been postulated that proteases are main drivers of BM loss, experiments in *C. elegans* did not reveal any proteases that are required for BM gap enlargement and furthermore showed that a reduction in BM deposition was not involved (Ihara et al. 2011). Instead, using optical highlighting of BM techniques, it was observed that the BM moves, sliding over the vulval and uterine cells to expand the BM opening. The force that drives BM sliding appears to be the rapid growth and division of the vulval cells (Fig. 6.4). An examination of BM remodeling during uterine-vulval attachment in 20 other species of nematodes (Dieterich et al. 2008;

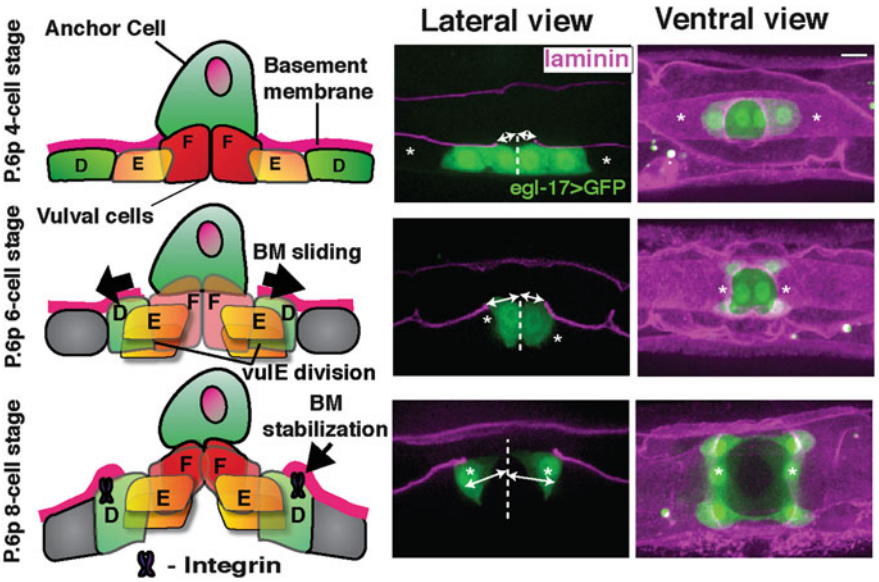

Fig. 6.4 BM sliding. *Top panel*: After the AC has invaded (P6.p 4-cell stage), the vulval cells begin to invaginate, grow, and divide, and the BM gap widens through BM sliding. The images on the right show lateral and ventral fluorescent images of the BM (laminin::mCherry) and the central vulval cells (vulE and vulF cells) expressing a cytoplasmic GFP driven by the *egl-17* promoter. The *dashed line* indicates the vulval midline, and "*asterisks*" marks the center of the vulD cell. *Middle panel*: By the P6.p 6-cell stage, the BM gap expands as it slides away from the AC and over the vulE cells, which have recently divided. *Bottom panel*: At the P6.p 8-cell stage, the vulF cells have divided, and the BM has expanded fully. The edge of the BM gap is stabilized by integrin upregulation on the vulD cells (note: the *egl-17* promoter drives expression in vulD cells at this time). *Scale bar* 5 μm (Reproduced from Matus et al. (2014) with permission from *Nature Communications*)

Matus et al. 2014) showed that a single AC initiates BM breach in all species with the subsequent BM gap stabilizing on vulD cells, the only vulval cell type that does not divide during vulval invagination (Fig. 6.4; Kiontke et al. 2007; Matus et al. 2014). These observations strongly suggest that cell divisions may promote BM sliding and nondividing cells might prevent it. Supporting this notion, live-cell imaging of BM sliding in *C. elegans* revealed that the centrally located vulE and vulF cells lose their attachment to the BM during their division and allow the BM to slide over these cells. Furthermore, experimentally halting the vulE and F cell divisions prevents BM sliding, while stimulating cell division in the vulD cells results in BM gaps moving beyond the dividing D cells. These observations in *C. elegans* and related nematodes indicate that cell division is a powerful regulator of BM sliding that has been maintained for hundreds of millions of years.

In addition to the controlled cell-cycle exit of vulD, increased integrin adhesion also plays an important role in stabilizing the position of the BM gap. Shortly after BM sliding stops on the vulD cell, the integrin adhesion receptor INA-1/PAT3

concentrates at the BM border (Fig. 6.4). Specific loss of integrin in the vulD cell results in further BM gap sliding, indicating that INA-1/PAT-3 (integrin) stabilizes the position of the BM gap border. The concentration of integrin is directed at least in part by increased levels of laminin at the edge of the BM gap, an increase caused by the AC as it pushes the BM aside during invasion (Hagedorn et al. 2013).

BM sliding may be a common morphogenetic mechanism to mediate tissue formation. For example, BM sliding has been observed during salivary gland growth in vertebrates and may allow bud expansion while restricting growth at the duct (Harunaga et al. 2014). Cell-BM shifts also occur to maintain tissues, as BM labeling and pulse chase experiments revealed that intestinal epithelial cells derived from the stem cell crypt slide along the BM toward the villus tips during differentiation to renew the gut epithelium (Clevers 2013; Trier et al. 1990). Additionally, as large gaps in the BM at tumor fronts have long been observed in cancer (Barsky et al. 1983; Frei 1962; Gabbert et al. 1985; Kobayashi et al. 1995; Rowe and Weiss 2008) and the loss of BM contact stimulates tumor cell invasion, it is possible that BM sliding plays a role in promoting the spread of cancer (Nguyen-Ngoc et al. 2012).

6.4 AC Invasion and SPARC: A Model for the Tumor Microenvironment

The tumor microenvironment is composed of the cells (e.g., fibroblasts, endothelial cells, and immune cells) and extracellular matrix of the surrounding tumor tissue and plays dynamic roles in tumor cell progression and metastasis (Joyce and Pollard 2009; Quail and Joyce 2013). AC invasion is regulated by numerous cues from the surrounding tissues, including integrin-BM interactions, an UNC-6 (netrin) chemotactic cue, and a diffusible cue that activates CDC-42 to promote invadopodia formation (Hagedorn and Sherwood 2011; Lohmer et al. 2016; Wang et al. 2014b). Notably, all of these molecules are key mediators of invasion and metastasis in cancer cells (Ko et al. 2014; Seguin et al. 2015; Stengel and Zheng 2011). This suggests that many of the environmental cues that control tumor invasion are conserved. AC invasion in *C. elegans* thus offers a potentially powerful in vivo experimental model to study the tumor microenvironment by altering the environment surrounding the AC and determining how these changes affect invasive behavior.

One example of an extracellular matrix protein misregulated in most tumor environments is the collagen-binding matricellular glycoprotein SPARC (Podhajcer et al. 2008). The overexpression of SPARC is strongly associated with tumor metastasis and poor prognosis in many aggressive cancers, including glioblastoma, pancreatic ductal carcinoma, breast ductal carcinoma, clear-cell renal cell carcinoma, melanoma, and prostate carcinoma (reviewed in Arnold and Brekken 2009; Nagaraju et al. 2014). Data from mouse models have confirmed that the overexpression of

SPARC increases metastatic potential (Minn et al. 2005; Ting et al. 2014) and in vitro assays support a role for increased SPARC in cancer invasion and migration in many different cancers (Briggs et al. 2002; Golembieski et al. 1999; Jacob et al. 1999; Kato et al. 1998; Ledda et al. 1997; Ting et al. 2014). SPARC has been implicated in regulating diverse cellular processes, including cell-matrix adhesion, growth factor activity, MMP expression, and extracellular matrix assembly and disassembly (Aguilera et al. 2014; Arnold and Brekken 2009; Barker et al. 2005; Harris et al. 2011; McClung et al. 2007; Sage et al. 1989; Shi et al. 2007). The numerous functions of SPARC and the challenge of studying invasion through BMs in vivo have made it difficult to determine how SPARC promotes invasive behavior.

In *C. elegans* the SPARC protein is made and secreted primarily by body wall muscles and accumulates in most BMs (Fitzgerald and Schwarzbauer 1998). To understand how the overexpression of SPARC may promote cell invasion, transgenic worms were generated expressing SPARC at two- to fivefold higher than normal levels (Morrissey et al. 2016). Elevated levels of SPARC did not alter the normal AC invasion program; however, increased SPARC fully restored AC invasion in mutants regulating diverse aspects of invasion—FOS-1A (breaching the BM/matrix, metalloproteinases), integrin (invadopodia and adhesion), vulval cue (CDC-42/invadopodia), and netrin (invasive protrusion; Fig. 6.5; Morrissey et al. 2016). These genetic interactions indicate that elevated SPARC is broadly pro-invasive and can compensate for the loss of multiple distinct pathways that promote invasion (Wang and Sherwood 2011).

How does SPARC so potently promote invasion in such a broad manner? Among the many functions of SPARC, likely the most ancient is its direct binding to collagens, where SPARC acts as a collagen chaperone that mediates the solubility and transport of collagens from sites of secretion to deposition (Martinek et al. 2002, 2007, 2008; Shahab et al. 2015). Work in the worm strongly implicates the misregulation of this collagen chaperone activity as underlying its pro-invasive function (Morrissey et al. 2016). First, the collagen-binding pocket of SPARC is essential for SPARC's pro-invasive functions (Sasaki et al. 1998). In addition, increased levels of SPARC dramatically decrease the levels and deposition rate of type IV collagen into the BM. Increased levels of SPARC delivered into the extracellular milieu from neurons (where neither SPARC nor type IV collagen are usually expressed) also promote invasion and decrease BM collagen. Finally, RNAi-mediated reduction of type IV collagen, a key structural and barrier component of BMs, recapitulates the broad pro-invasive functions of SPARC. Together these results suggest that elevated levels of SPARC may increase collagen solubility to such an extent that it inhibits collagen deposition into BMs. The decrease of collagen in BMs likely reduces the barrier properties of BMs, thus allowing even poorly invasive ACs to invade through these BMs (Fig. 6.6).

The observations of SPARC in the worm may account for the broad pro-invasive function of SPARC in numerous types of cancer. Further, these studies could explain the finding that SPARC overexpression by either the tumor or the surrounding tissue promotes metastatic progression (Barth et al. 2005; Iacobuzio-Donahue et al. 2002; Kato et al. 1998; Rich et al. 2003; Rodriguez-Jimenez et al. 2007; Sato

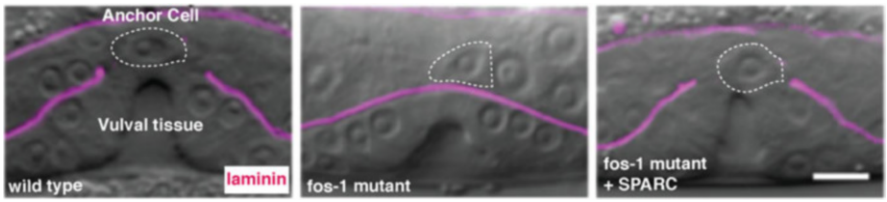

Fig. 6.5 Overexpression of SPARC promotes AC invasion. *Top panel*: A wild-type AC that has successfully invaded by the P6.p 8-cell stage. BM visualized by fluorescently labeled laminin, whose levels are not altered by SPARC. *Middle panel*: AC invasion has been blocked by a mutation in *fos-1*, a major regulator of invasion. *Bottom panel*: The overexpression of SPARC restores the ability of the AC to invade in the *fos-1* mutant. *Scale bar* 5 μm (Figure reproduced from Morrissey et al. (2014) with permission)

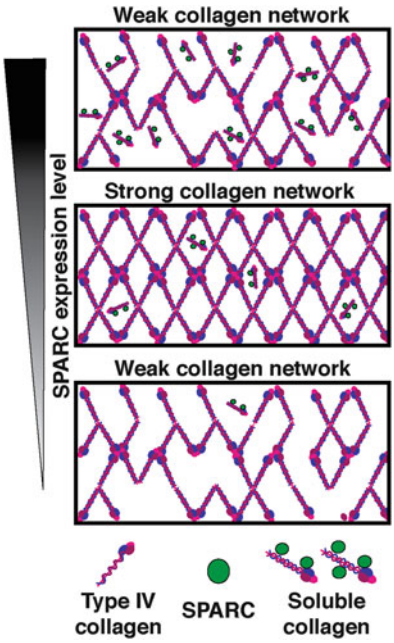

Fig. 6.6 The levels of SPARC expression regulate the BM type IV collagen lattice. A schematic diagram depicting SPARC function. SPARC is a collagen chaperone that maintains extracellular collagen solubility. *Bottom panel*: When SPARC levels are low, there is not enough soluble type IV collagen to build a strong collagen BM lattice. *Middle panel*: At normal levels, SPARC facilitates efficient type IV collagen solubility to allow the transit of collagen from sites of secretion to assembly locations for strong lattice construction. *Top panel*: When overexpressed, SPARC maintains collagen solubility in the extracellular fluid and inhibits efficient collagen deposition in type IV collagen BM networks, resulting in a weak lattice

et al. 2003; Schultz et al. 2002) as secretion of SPARC from any tissue in the vicinity would reduce type IV collagen levels in the BM surrounding the growing tumor or tumor vasculature. The findings of SPARC function in the worm are also consistent with observations in a mouse model of breast cancer metastasis, indicating that SPARC is not sufficient to drive metastasis but dramatically enhances tumor invasion (Minn et al. 2005). Given the complexity of the tumor-stromal interactions, understanding how alterations in the tumor microenvironment regulate invasive behavior is a daunting task. These studies with SPARC illustrate how AC invasion is not only a model to elucidate the normal invasion program but is also emerging as an effective experimental paradigm to determine how alterations in the tumor microenvironment regulate invasion and metastasis.

6.5 Summary and Perspective

Studies of AC invasion and uterine-vulval attachment in the worm have advanced our understanding of the transcriptional networks, signaling pathways, cellular dynamics, and extracellular matrix removal mechanisms that allow invasive cells to transmigrate and widen BM gaps. Notably, these studies have uncovered important new mechanisms underlying the invasion and BM removal that have acute relevance to therapeutic strategies used to block invasive behavior in cancer, such as a requirement for G1 cell-cycle arrest in invasive cells and the ability of cells to remove BM barriers by physical displacement. Further, AC invasion is emerging as a promising model to study how alterations in the tumor microenvironment affect cell invasive behavior. Yet, many important questions in understanding how cells transmigrate BM barriers remain, including the role of proteases in cell invasion and elucidating the complete transcriptional network that programs invasiveness. Furthermore, there is clear evidence that biomechanical properties of tissues and interstitial type I collagen matrices play important roles in cell invasion in cell culture and in tumor invasion (Kai et al. 2016). We do not yet understand what role, if any, BM stiffness plays during BM invasion or how the BM's physical properties might be altered during invasion. With the recent development of single-cell profiling techniques, CRISPR-Cas9 genome editing, FRET-based force sensors, and sensitized screening in the worm (Dickinson et al. 2013; Gayrard and Borghi 2016; Spencer et al. 2014), AC invasion is poised to remain at the leading edge of understanding how cells transmigrate and remove BM barriers.

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