Advances in Experimental Medicine and Biology 1234

Alexander Birbrair *Editor*

Tumor Microenvironment

Non-Hematopoietic Cells

Springer

Advances in Experimental Medicine and Biology

Volume 1234

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2018 Impact Factor: 2.126.

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-37183-8 ISBN 978-3-030-37184-5 (eBook)
https://doi.org/10.1007/978-3-030-37184-5

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

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

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

Nikitha K. Pallegar and Sherri L. Christian from Memorial University of Newfoundland discuss the role of adipocytes in the tumor microenvironment. Fabio Corsi and colleagues from the Università degli studi di Milano describe fibroblasts in the tumor microenvironment. Lan Coffman and colleagues from the University of Pittsburgh School of Medicine compile our understanding of mesenchymal stem cells in the tumor microenvironment. Hidenori Shiraha and colleagues from Okayama University Faculty of Medicine update us with what we know about hepatic stellate cells in liver tumor. Divya Thomas and Prakash Radhakrishnan from the University of Nebraska Medical Center focus on the pancreatic stellate cells, as key orchestrators of the pancreatic tumor microenvironment. Jolanta Niewiarowska and colleagues from

Medical University of Lodz summarize current knowledge on endothelial cells in the tumor microenvironment. Sophia Ran and Lisa Volk-Draper from Southern Illinois University School of Medicine address the importance of lymphatic endothelial cell progenitors in the tumor microenvironment. Finally, Takuichiro Hide and Yoshihiro Komohara from Kitasato University School of Medicine give an overview of oligodendrocyte progenitors in the tumor microenvironment.

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

Belo Horizonte, Minas Gerais, Brazil Alexander Birbrair

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Adipocytes in the Tumour Microenvironment

1

Nikitha K. Pallegar and Sherri L. Christian

Abstract

Adipose tissue contribution to body mass ranges from 6% in male athletes to over 25% in obese men and over 30% in obese women. Crosstalk between adipocytes and cancer cells that exist in close proximity can lead to changes in the function and phenotype of both cell types. These interactions actively alter the tumour microenvironment (TME). Obesity is one of the major risk factors for multiple types of cancer, including breast cancer. In obesity, the increase in both size and number of adipocytes leads to instability of the TME, as well as increased hypoxia within the TME, which further enhances tumour invasion and metastasis. In this chapter, we will discuss the diverse aspects of adipocytes and adipocyte-derived factors that affect the TME as well as tumour progression and metastasis. In addition, we discuss how obesity affects the TME. We focus primarily on breast cancer but discuss what is known in other cancer types when relevant. We finish by discussing the studies needed to further understand these complex interactions.

Keywords

Tumour microenvironment · Adipocytes · Obesity · Paracrine/autocrine signaling · Adipokines · Lipid metabolites · Breast cancer · Metastasis · Epithelial to mesenchymal transition · Mesenchymal-to-epithelial transition · Extracellular matrix (ECM) · Hypoxia · Chronic inflammation · ECM remodeling

1.1 The Tumour Microenvironment

Genetic or epigenetic instability in cancer cells leads to activation of signaling networks that, together with neighbouring cells and extracellular matrix (ECM) proteins, promotes the generation of a tumour microenvironment (TME) that specifically supports tumour growth. The TME is comprised of ECM proteins and several stromal cell types such as endothelial cells, fibroblasts, immune cells, pre-adipocytes, adipocytes, and inflammatory cells that play a crucial role in tumour growth and development [1] (Fig. 1.1). Abnormal conditions, like those seen in obesity, can contribute to breast cancer (BC) progression by changing the TME [2].

Cancer metastases account for 90% of all human cancer-related deaths, including in BC [3]. The metastatic cascade is a very complex and

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_1

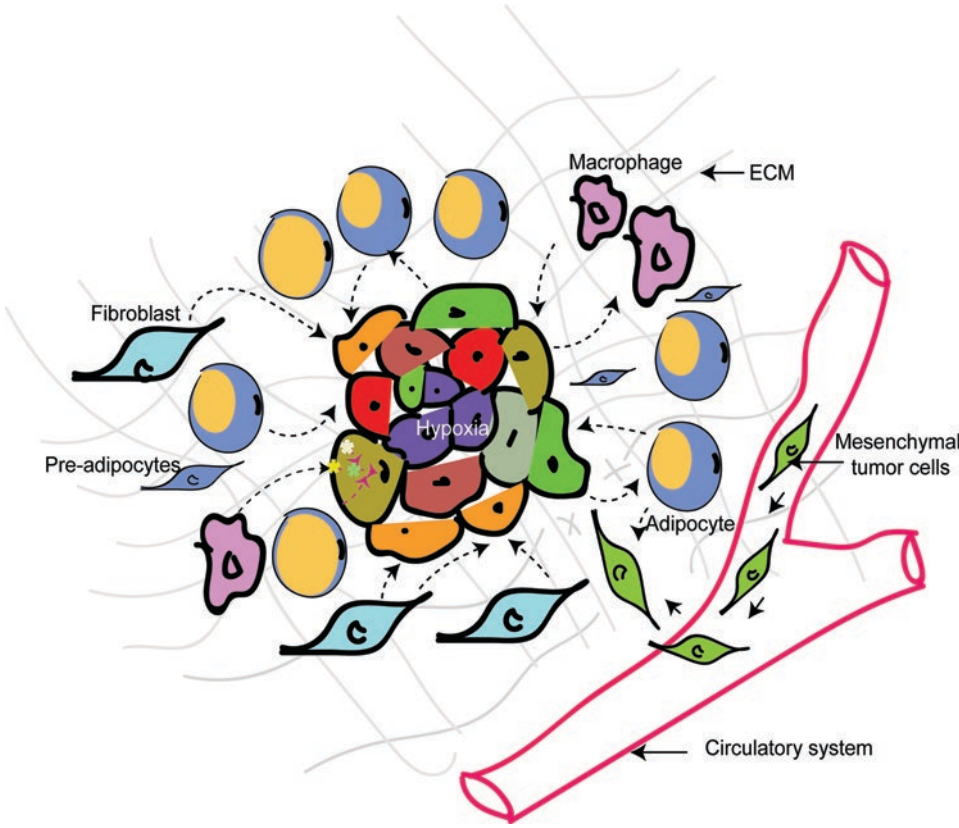

Fig. 1.1 Schematic illustration of the tumour microenvironment showing the interaction between tumour cells, non-cancerous stromal cells, and the surrounding ECM. The tumour consists of a heterogeneous population of cells with varied mutational burden between cells. Coloured asterisks (∗) indicate the complexity of cross-talk between different signaling pathways when cells have multiple mutations. The lipid-engorged adipocytes are shown interacting with cancer cells, and promoting the denaturation of the ECM at the site of colonization. Dashed lines with arrows indicate paracrines interaction between cells. Different colours of cells within the tumour indicate the tumour heterogeneity that occurs due to acquisition of diverse mutations within each cell or cell population

poorly understood process. It includes a series of steps that starts with tumour progression, tumour invasion, matrix remodeling, and intravasation, followed by extravasation, and ending with colonization of the tumour cells at distant sites (Fig. 1.2). During metastasis, cancer cells undergo dissemination from the primary tumour and can achieve migration via an epithelial-to-mesenchymal transition (EMT), followed by a colonization of tumour at secondary site via mesenchymal-to-epithelial transition (MET). All of these events alter the TME at both primary and secondary tumour sites.

1.1.1 EMT

Transformation of tumour cells, accompanied by the generation of a pro-inflammatory tumour-associated stroma, induces invasion via EMT. Initiation of EMT is regulated by intrinsic factors such as activation of signaling pathways, transcription factors, microRNAs, or epigenetic modulation that are in turn influenced by extrinsic factors including tumour-stroma interactions [4]. Stromal cells such as adipocytes can induce the expression of mesenchymal markers and promote invasiveness of BC cells, suggesting a pro-

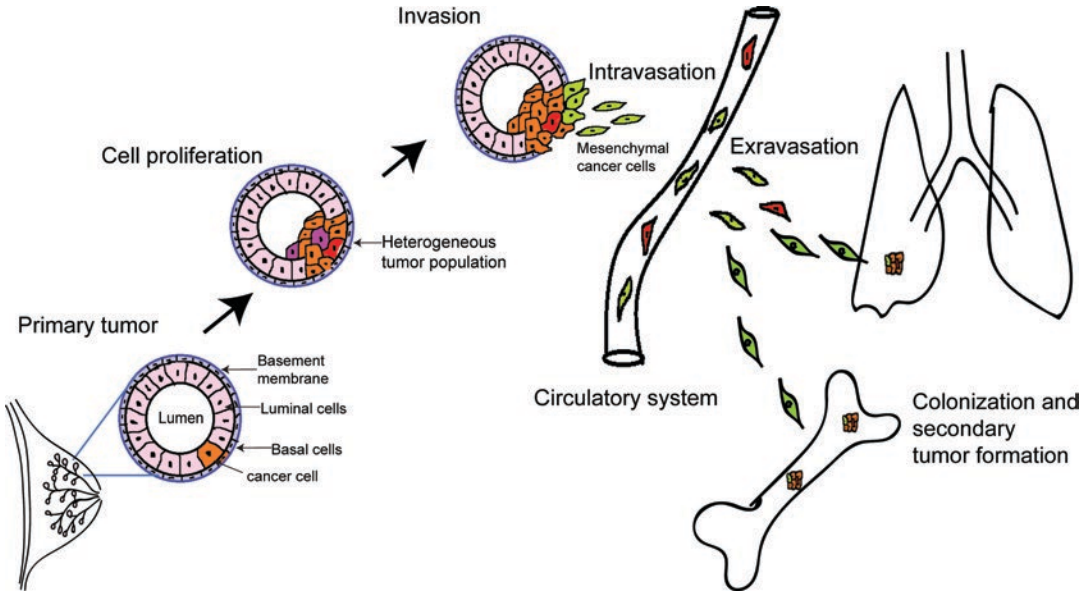

Fig. 1.2 Stages of BC initiation, progression, and metastasis. Increased genetic and epigenetic instability, accompanied by a pro-inflammatory TME leads to tumorigenesis and increases in cell proliferation (orange). Accumulation of mutations leads to heterogeneous tumour population including cancer stem cells (red), cancer cells with different mutational (dark purple) burden. Epithelial cells change to mesenchymal cells (green) to invade the basement membrane and remodel the extracellular matrix. Mesenchymal tumour cells intravasate into the circulatory system, migrate to distant sites, and then extravasate into the tissue parenchyma to subsequently colonize and form tumours at secondary sites such as lung or bone marrow [111]

EMT regulation [5]. Adipocytes from visceral white adipose tissue (WAT) have enhanced effects on the EMT of BC cells compared to those from subcutaneous WAT [6]. Stromal cells secrete proteins such as transforming growth factor (TGFβ), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) that can induce EMT as well as inducing proliferation, protection from apoptosis, and angiogenesis [7, 8]. The signaling pathways that are associated with induction of EMT include TGFβ, wingless/integrated (Wnt)/β-catenin, and Notch pathways [4]. These pathways activate the master regulators of EMT, which include the transcription factors (TF) Snail, Slug, zinc finger E-box binding homebox 1 (ZEB 1), ZEB2, goosecoid, forkhead box C1 (FOXC1), FOXC2, and twist family basic helix-loop-helix (bHLH) TF (TWIST). EMT-TFs transcriptionally downregulate the expression of adherens junction and integrin proteins, which allows transformed cells to lose polarity and dissociate from adjacent cells and the basal membrane [9, 10]. The E-cadherin promoter is repressed by Snail, Slug, and ZEB2 directly and by TWIST1, FOXC2, and ZEB1 indirectly, which disrupts cell polarity and maintains the mesenchymal phenotype to promote EMT [9, 11]. TWIST1 can promote transformation of normal mammary epithelial cell into mesenchymal-like cells that have increased expression of vimentin, N-cadherin, and fibronectin [12].

Once EMT is initiated, cells lose polarity and become mobile, whereupon they can invade the basement membrane and degrade the ECM. Snail1 and Snail2 expression in BC cells increase membrane type 1-matrix metalloproteinase (MT1-MMP), MT2-MMP, MT4-MMP, and MMP2 expression which further leads to the degradation of basement membrane and allows subsequent tumour metastasis [13]. Adipocytes have a crucial role in modifying ECM by secreting MMPs into TME that further enhances invasion by cancer cells [14]. EMT-TFs

induce the formation of specialized structures called invadopodia, which invade local ECM. TWIST1 and TGFβ enhance invadopodia formation, which actively promotes degradation of the matrix [15]. Moreover, MMPs and other chemokines released from epithelial cells and inflammatory cells in the TME disrupt the basement membrane and promote focal degradation of ECM proteins such as collagen and laminin [16].

Cancer cells undergo intravasation to invade into the lymphatic and blood circulatory systems. EMT markers, matrix remodeling proteins, and angiogenic factors have an essential role in intravasation of cancer cells. In pancreatic cancer, increased ZEB1 expression enhances migration through the endothelial barrier followed by metastatic colonization [17]. Activation of membrane bound proteins, MT1-MMP and MT2-MMP but not MMP, allows cancer cells to come in contact with endothelial cells and then intravasate into the vasculature [13]. To disrupt the vascular integrity during both intravasation and extravasation, cancer cells express vascular endothelial growth factor (VEGF), MMPs, and a disintegrin and metalloproteinase (ADAM) [18].

Cancer cells disseminate as single cells or clusters, both retaining mesenchymal properties. Circulating tumour cells (CTCs) retain these mesenchymal properties via activation of the TGFβ pathway [19]. Moreover, in a mouse mammary tumour model, increases in the protein expression of the EMT marker TWIST1 were found during early stages of tumour formation, and cells remained in a mesenchymal state until they reached the bone marrow [20].

1.1.2 MET

The reverse process of EMT is known as MET, whereby the mesenchymal CTCs extravasate into the distant tissue parenchyma and dedifferentiate into an epithelial phenotype to form a secondary tumour (Fig. 1.2). The mechanisms involved in organ-specific extravasation of CTCs are still elusive. According to previous studies, many factors such as the circulatory system, microenvironment, adaptability to the tissue parenchyma, and tumour initiating ability have an impact on colonization of CTCs at a specific site. In some cancers, like colorectal cancer, metastasis in the liver is explained by the draining of blood in the portal vein into the liver from the colon [21]. When CTCs enter the microenvironment of the tissue parenchyma at a secondary site, they encounter ECM and stromal cells including fibroblasts, adipocytes, and inflammatory cells. Co-culture of adipose tissue-derived stem cells upregulates E-cadherin expression and downregulates vimentin and N-cadherin expression in liver cancer cells [22], suggesting an MET shift. However, how these factors are involved in macro- or micro-metastases induction is unclear.

During EMT, cell division is repressed by Snail1 and ZEB2 via inhibition of cyclin D activity, which slows down cell proliferation and promotes differentiation [15]. However, during MET, epithelial properties such as proliferation and adhesion are regained by cancer cells [23]. Several pathways such as the Ras/extracellular signal regulated kinases (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt, and Wnt signaling pathways in BC cells induce an epithelial phenotype [21, 24]. TFs such as Snail and TWIST that promote EMT are also repressed during metastasis which further assists in suppressing the mesenchymal phenotype and regaining epithelial phenotype. In various studies, it has been proven that mesenchymal cells acquire epithelial properties after metastasis as recognized by expression of E-cadherin [21, 25, 26]. Forced expression of E-cadherin can, in fact, induce MET in prostate cancer cells [27]. Moreover, cancer cells use E-cadherin to connect with local normal epithelial cells and establish tumour formation at secondary sites.

1.2 White Adipose Tissue

WAT is present at multiple sites in the body, which allows it to interact with many different types of solid tumours. WAT is histologically characterized as soft connective tissue. In addition to providing an energy source, WAT is an

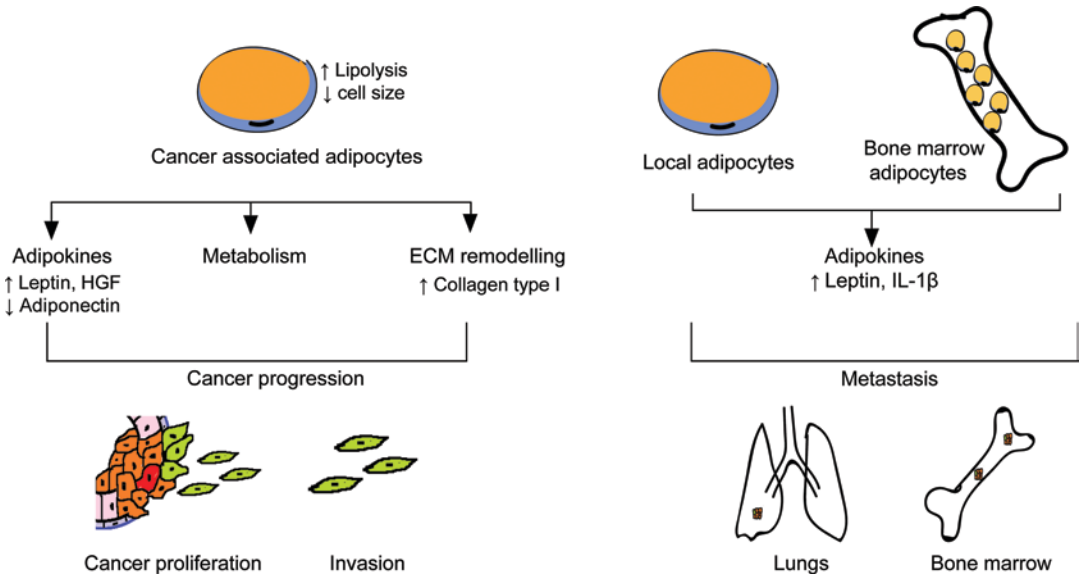

Fig. 1.3 Schematic illustration of role of adipocytes in different stages of cancer. Cancer-associated adipocytes (CAA) contribute to tumour progression via secretory factors such as adipokines, via alterations to cancer cell metabolism, and via remodeling the ECM. Metabolites from the lipolysis of CAA also contribute to cancer cells proliferation. Adipocytes under obese conditions secrete increased levels of adipokines such as leptin and hepatocyte growth factor that promote inflammation. Moreover, increases in adipocyte activity and size leads to an accumulation of collagen that causes stiffening of the microenvironment. CAA can also induce systemic and local changes leading to increased levels of pro-inflammatory adipokines that contribute to metastasis to organs such as the lungs and liver. Local adipocytes in bone marrow also release adipokines such as leptin and IL-1β that promotes tumour cell homing

active endocrine organ, a function that further regulates tumour growth, invasion, and metastasis via the production of metabolites, hormones, and cytokines (adipokines) [28]. Some of the most prominent interactions between solid tumours and WAT can be seen in the breast. Breast tissue is 90% WAT with permanent interactions between epithelial cells and adipocytes. WAT is also critical for normal mammary gland development [29]. Moreover, adipocytes have both mechanical and biochemical interactions with BC cells that can regulate tumour progression [30].

WAT is known to contribute to progression, invasion, and metastasis of cancer cells (Fig. 1.3). The interaction between cancer cells and adipocytes leads to the increased activity of adipocytes. For example, adipocytes activated by ovarian cancer cells show differential gene expression and changes in function that have been shown to contribute to tumorigenesis [31]. In addition, under obese conditions, adipocytes show elevated functional activity, leading to increases in factors related to pro-inflammation, hypoxia, angiogenesis, and ECM remodeling [32] (Fig. 1.1). The adipocyte secretome is also modified when co-cultured with cancer cells, where an upregulation of MMP-11, osteopontin, TNF-α, and IL-6 has been observed [33]. Moreover, adipocyte cell size and cell number is decreased in the vicinity of the tumour compared to adipocytes that are distant from the tumour [34]. BC cells co-cultured with adipocytes in a transwell system also show reciprocal effects on adipocytes, where BC causes a decrease in lipid droplet (LD) number in adipocytes [35]. Moreover, there can be an increase in fibroblast-like cells at the tumour site, such as seen in melanoma, suggesting that adipocytes may be undergoing active dedifferentiation due to in response to interactions with tumour cells [36].

WAT is composed of mature adipocytes, and cells found in the stromal vascular fraction

(SVF), which includes adipocyte-derived stem cells, pre-adipocytes, immune cells, pericytes, endothelial cells, and fibroblast cells [37]. Mature adipocytes contribute to 80% of the WAT secretome and shares 60% of these proteins with the SVF [38]. WAT as an endocrine organ secretes a variety of factors such as metabolites, enzymes, hormones, growth factors, and cytokines called as adipokines involved in communication with the surrounding environment for growth and development. So far, more than 100 adipokines have been evaluated, of which only a few are heavily studied, such as leptin, adiponectin, reisistin, visfatin, insulin-like growth factor (IGF), HGF, TGF, tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [38].

1.2.1 Leptin

Leptin regulates energy balance by suppressing hunger to inhibit food intake [39]. Under obese conditions, serum leptin concentrations are increased, but often the receptors for leptin become dysfunctional and unresponsive to leptin. The presence of dysfunctional leptin receptors leads to excess food intake and is associated with obesity [40]. BC cells express leptin receptors; thus, leptin can induce proliferation and growth of BC cells. Leptin also induces pro-inflammatory responses by activating monocytes and macrophages and so contributes to chronic inflammation seen with obesity [41]. In vitro experiments suggest that increases in leptin concentration elevates the proliferation of both oestrogen receptor (ER)-positive and ER-negative BC cell lines via Janus/kinase2 (Jak2) and PI3K signaling [42] and acts as growth factor that enhances invasive ductal carcinoma and invasive lobular carcinoma progression in vivo [43]. In addition, leptin regulates multiple properties of cell growth such as the cell cycle, signaling pathways, and apoptosis, all of which contribute to BC progression [44, 45]. Lastly, silencing leptin receptor expression in triple-negative breast cancer (TNBC) cells leads to MET with increased E-cadherin expression and decreased vimentin expression suggesting that leptin may also have a role in maintaining the mesenchymal state in TNBC cells [46].

1.2.2 Adiponectin

Adiponectin plays an important role in regulation of lipid and glucose metabolism [47]. In obesity, adiponectin levels are reduced, which leads to an accumulation of lipids and glucose that in turn promotes insulin resistance and obesity. Adiponectin has anti-inflammatory properties that modulate the inflammatory functions of immune cells and promote activation of anti-inflammatory macrophages [48]. Notably, adiponectin suppresses BC growth and invasion while enhancing apoptosis [49] as well as inhibiting PI3K activation and suppressing BC cell proliferation [50]. Cancer-associated adipocytes (CAA) have been found to secrete reduced levels of adiponectin [30]. Interestingly, a high leptin to adiponectin ratio has been linked to increased risk of TNBC progression [51], suggesting that the relative ratios of these cytokines may drive BC progression.

1.2.3 Other Adipokines

IL-6 secreted by adipocytes not only regulates lipogenesis locally but also acts systemically [52]. Obesity leads to an increase of IL-6 in circulation, further adding to inflammation [48]. Increased levels of IL-6 are correlated with poor prognosis, progression, and migration of ER-positive BC [53]. TNF-α is an inflammatory cytokine and in WAT is secreted primarily by macrophages. TNF-α is increased in TME of obese humans due WAT inflammation, and an increase in TNF-α inhibits apoptosis of TNBC cells [54, 55]. Resistin is another adipokine shown to promote tumour growth; however, there is no direct link between resistin, obesity, and BC shown thus far [56]. Autotaxin (ATX) is also secreted from adipocytes, and disruption of adipocyte specific ATX in mice fed a high-fat diet leads to increases in fat mass showing that ATX is a negative regulator of fat mass expansion [57].

ATX-lysophosphatidate signaling activates several cellular processes resulting in the increased invasiveness and motility of BC cells [58]. Obesity is associated with increased levels of circulating insulin like growth factor-1 (IGF-1). BC cells express IGF-1 receptors, and binding of IGF-1 activates PI3K and MAPK pathways leading to cell proliferation of tumour cells [59–61]. Similarly, serum levels of HGF are elevated by adipocytes during obesity, and its receptor, c-Met, is expressed on BC cells; therefore, increased expression of HGF promotes c-Met-induced cell proliferation and subsequent tumour progression [62, 63].

1.2.4 Lipid Metabolites

Metabolic reprogramming is considered an emerging hallmark of cancer [64, 65]. Cancer cells generate adenosine triphosphate (ATP) from aerobic glycolysis instead of mitochondrial oxidative phosphorylation; this change in metabolism is known as the Warburg effect [66]. The "reverse Warburg effect" is observed when cancer cells use the energy generated from stromal cells in the tumour microenvironment [67]. In addition to glucose, cancer cells take up free fatty acids and glycerol as a source of energy from stromal adipocytes. Moreover, tumour cells rely on stromal sources for metabolic substrates such as lactate, glutamine, and fatty acids via stimulation of glycolysis and lipolysis pathways in stromal cells [68]. Uptake of glucose metabolites in cancer progression is well known [69]; however, the involvement of lipid metabolites has been less well defined.

Reprogramming of lipid metabolism is part of the alterations in energy metabolism that occurs in cancer cells. Adipocytes regulate energy balance in the whole organism by storing triglycerides via lipogenesis and by the production of diacylglycerol, monoacylglycerol, and free fatty acids via lipolysis within a cell. Highly proliferative cancer cells meet their energy requirements by synthesizing lipids and cholesterol endogenously through lipogenesis or by obtaining them from the TME by stimulating lipolysis in adipocytes [65, 70]. To understand the adipocyte-tumour metabolic crosstalk better, there has been an initiative for in vitro co-culture studies of BC cells and adipocytes or adipocyte-conditioned medium. Co-culture of adipocytes and BC cells increases lipolysis in adipocytes via hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), resulting in release of free fatty acids that were transferred into adjacent BC cells as an energy source [71]. Moreover, a decrease in lipid droplet size and number has been reported in CAA [35]. Free fatty acids can be used for mitochondrial β-oxidation or as metabolic substrates that supports cancer proliferation and migration. Increases in lipid metabolites are reported for many cancers such as breast, prostate, glioblastoma, and hepatocellular carcinoma [72]. Fatty acid binding protein (FABP) family proteins are expressed on cells involved in active lipid metabolism. The FABP protein FABP4, which is involved in transport of fatty acids, is increased during BC progression [28].

Additionally, cancer cells utilize lipids for cell membrane formation, generation of lipid-derived bioactive molecules, and generation of exosomes. Free fatty acids and glycerol released from lipolysis can be used for biosynthesis of membrane lipids during BC proliferation [73]. Bioactive lipids such as steroid hormones, diacylglycerol, eicosanoids, phospholipids, and sphingolipids also participate in metabolic reprogramming of cancer cells [65]. The fatty acid receptor CD36 is involved in initiation of metastasis in breast-derived tumours and is associated with poor prognosis [74]. Adipocyte-derived exosomes, also known as adiposomes, can stimulate cell invasion and migration in melanoma cancer cells [75].

1.2.5 ECM

Within the WAT, adipocytes secrete a wide variety of ECM components needed for mechanical support that can also affect cancer progression [76, 77]. Adipocytes are surrounded by basement membrane with collagen type VI and laminin as the major constituents [78]. Collagen type VI

promotes the growth and survival of BC cells via NG2/chondroitin sulphate proteoglycan receptors [79], while the endotrophin component of the collagen VI protein promotes EMT and initiates metastasis [80]. Adipocytes also secrete matrix metalloproteinases such as MMP1, MMP7, MMP10, MMP11, and MMP14 which participate in remodeling the ECM [14]. MMPs are also known as important regulators of tumour invasion, allowing cancer cells to migrate through the ECM. For example, expression of MMP11 induced in adipocytes by hepatocarcinoma cells promotes ECM remodeling and tumour invasion [81]. Moreover, MMP11 suppresses adipocyte differentiation and enhances dedifferentiation, leading to an increase in fibroblast cells in glioblastoma and osteosarcoma, which further amplifies tumour invasion [30], but the role of MMP11 in BC is unknown.

1.3 Obesity and Breast Cancer

According to the World Health Organization (WHO), over 2 billion people in the world are overweight or obese, and it is estimated that by 2030 > 3.3 billion (57.8%) of the adult world population will be overweight or obese [82, 83]. Obesity is now considered as one of the most important risk factors contributing to overall disease burden in the world [84].

Over 40% of cancer patients are classified as overweight or obese [85, 86]. Obese women with BC have larger tumours and enhanced metastasis that contributes to a 30% increased risk of death [87–89]. Obese post-menopausal women are at high risk for ER-/PR-positive BC, whereas obese pre-menopausal women are at higher risk of developing TNBC compared to lean women [90]. Moreover, obese patients do not respond to therapy as well as lean patients, particularly when diagnosed with TNBC, also contributing to the overall worse prognosis [90, 91].

In comparison to subcutaneous WAT, visceral WAT is more metabolically active, with increased accumulation of inflammatory cells and cytokines [92]. Women with visceral obesity have a higher risk of BC occurrence than women with subcutaneous obesity [93]. Both obesity and TNBC are associated with development of visceral metastases [94, 95]. In obese patients with ovarian or prostate cancer, an increase in the number of bone marrow adipocytes is correlated to increased skeletal metastasis [96, 97].

Obesity is characterized by the enlargement of WAT depots with excess engorgement of lipids in adipocytes. In addition, excess intake of energy leads to increase in adipocyte size (hypertrophy) and eventually the number of adipocytes (hyperplasia). In early stages of WAT expansion, adipocyte hypertrophy generates a local WAT hypoxia that contributes to systemic changes such as increases in adipokines, secretion of inflammatory cytokines, lipid metabolites, fibrosis, and CSC, which can contribute to BC progression [2]. These systemic changes further reduce the metabolic flexibility of adipocytes, therefore increasing the rate of apoptosis and ultimately accumulating more inflammatory cells in WAT. Moreover, chronic hypoxia observed in obese WAT results in chronic inflammation, ER stress, and an alteration in TME which leads to BC progression. Hypoxic conditions trigger the activation of hypoxia-induced factor 1 (HIF-1) in adipocytes which is associated with poor prognosis in obese BC patients [98]. In addition to hypoxia, the increase in adipocyte size causes a stiffer ECM to be deposited by adipocyte stromal cells in obese BC patients [99]. The chronic inflammation that occurs in obese WAT leads to secretion of cytokines such as IL-6 and TNF-α that are known to affect cancer progression. When they occur together, obesity and TNBC are the worst combination for a patient's outcome.

Both obesity and cancer change the gene expression and functional characteristics of adipocytes with reciprocal effects on cancer progression. Adipocytes communicate with cancer cells and can participate in the initiation of metastasis via secretory factors and ECM remodeling (Fig. 1.3). It is known that local adipocytes can trigger BC metastasis to the liver and lungs via paracrine signaling. Importantly, the presence of adipocytes at distant sites can intensify tumour metastasis, as in the case of bone marrow adipocytes [96, 97]. Bone marrow adipocytes secrete

IL-1β, which promotes the homing of BC cells to the bone [100]. These conditions worsen in patients with obesity, where increased pro-inflammatory factors, adipokines, and changes in the connective tissue composition can promote invasion, migration, and metastasis. Therefore, adipocytes located at both the primary site and secondary sites can play a crucial role in the process of BC metastasis.

1.4 Future Perspectives

Unravelling the complex interactions between the adipocytes and cancer cells requires the use of model systems that better recapitulate the in vivo TME. Structurally, the normal human mammary gland is embedded in ECM, whereas the mouse mammary gland has a greatly reduced ECM component; thus, mice do not adequately model the human condition with respect to the ECM: adipocyte ratios [101]. Traditional 2D in vitro cell culture systems lacks the 3D organization of cells between each other or with the ECM that is occurs in organs and tissues in vivo [102]. In contrast, 3D cell culture systems better mimic in vivo conditions and can bridge the gap between in vitro systems and human patient trials [103–105]. Cells grown in 3D obtain a more physiological morphology, displaying aggregate structures or spheroids with prevalent cell junctions. Moreover, cells in 3D obtain phenotypic heterogeneity with a varied cell proliferation rate, gene expression, and differentiation within one population [106]. Exposure to nutrients, growth factors, or drugs is also heterogeneous where cells on the outer side of a spheroid are more exposed compared to cells in the inner core, which is more similar to in vivo conditions. In addition, cells in 3D have greater viability and less susceptibility to external factors and show increased resistance to drug-induced stimuli [107, 108]. Lastly, both MET and EMT involve interactions with the ECM that are recapitulated in 3D, but not 2D, culture systems [109, 110]. We have found that adipocytes promote MET in mesenchymal TNBC cells when cultured in the 3D environment unlike studies performed in 2D [109], further demonstrating that the 3D environment fundamentally changes the responsiveness of cells. Development of additional in vitro models is critical to unravelling the multiple interactions in the TME and to identify factors that may be targeted therapeutically to reduce cancer progression, including metastasis.

1.5 Summary

Overall, growing evidence suggests that adipocytes are active players in modifying the TME in a way that it promotes cancer progression and metastasis. Importantly, crosstalk between adipocytes and cancer cells has reciprocal effects on adipocytes and the secretome that shapes the TME. Moreover, paracrine or autocrine signaling by adipocytes influences cancer development at both primary and secondary sites. Adipocytes can regulate the expression of EMT/MET markers at different stages of metastasis [5, 109]. Under obese conditions, the interactions between adipocytes, TME, and cancer cells can contribute to worst prognosis in cancer patients. Hypoxia, chronic inflammation, and increased ECM stiffness that occur in obesity are the major alterations of the TME that can drive tumour progression. Adipocytes not only provide metabolites and energy sources to cancer cells but can also protect the cancer cells from different therapies. Understanding the interactions between adipocytes and the TME is of fundamental and clinical interest that can improve the treatment strategies for obese cancer patients.

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Fibroblasts in the Tumor Microenvironment

2

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Abstract

The implications of a tumor microenvironment in cancer initiation and progression have drawn interest in recent years. Within the tumor stroma, fibroblasts represent a predominant cell type and are responsible for the majority of extracellular components within the tumor microenvironment, such as matrix and soluble factors. A switch from quiescent fibroblasts to cancer-associated fibroblasts triggers a large variety of pro-tumorigenic signals that support tumor progression and shape the surrounding pathological stroma, with the remodeling of tissue architecture and repression of the local immune response. The heterogeneous nature of cancer-associated fibroblasts and their multiple functions are subject of active research as they could represent promising targets for cutting-edge therapeutic approaches to cancer and the tumor microenvironment.

Keywords

Cancer-associated fibroblasts · Epithelial-to-mesenchymal transition · Tumor microenvironment · Pro-tumorigenic cytokines · Extracellular matrix remodeling · Tumor neoangiogenesis · Immunosuppression · Cancer-stroma crosstalk · Chemoresistance · Targeted therapy · Cancer treatment

2.1 Switching the Focus from Tumor to Tumor Microenvironment

The biological implications of the tumor microenvironment (TME) on cancer progression and its spreading have begun to be suggested over the past few years. Several studies have demonstrated that TME is not just a silent bystander, but rather an active promoter of cancer progression. A *milieu* of immunosuppressive T-reg lymphocytes, tumor-associated macrophages, fibroblasts, and adipocytes makes up the TME, providing a real sanctuary for cancer [48]. In particular, cancer-associated fibroblasts (CAF) are key components of the TME, closely supporting cancer by

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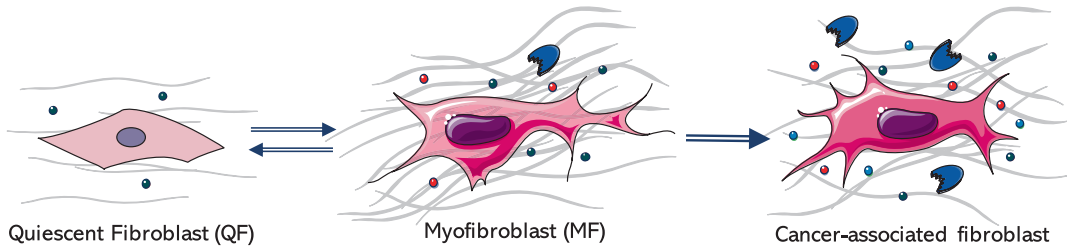
A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_2

secreting mitogenic growth factors such as a fibroblast growth factor (FGF) or the insulin-like growth factor 1 (IGF-1) [4]. Furthermore, CAF are centrally involved in the NF-kB inflammatory signaling pathway which promotes tumor progression, also stimulating neo-angiogenesis. The transforming growth factor beta (TGF-β) deriving from CAF induces the epithelial-to-mesenchymal transition (EMT), which is considered the key process in cancer invasion and distant spread due to the acquisition of mesenchymal stem cell features. Not only are CAF involved in such a complex crosstalk between cancer cells and TME, they are also structurally fundamental for a cancer-supporting TME. Indeed, CAF produce extracellular matrix (ECM) proteins, which are responsible for the desmoplastic reaction at the edges of a tumor and within cancer cells, protecting them from antitumor immune responses and chemotherapeutics. Recently, the interest of researchers and clinicians has focused on CAF, considered to be key mediators of cancer-stroma crosstalk, and a promising target for novel therapeutic approaches toward TME in cancer treatment.

2.2 The Heterogeneous Nature of Cancer-Associated Fibroblasts

2.2.1 Origins and Functions of CAF

Fibroblasts are the most abundant stromal cells in the TME, accounting for up to 80% of the tumor mass in certain solid tumors characterized by a desmoplastic reaction [120]. They are particularly important because of their continuous and complex crosstalk with cancer cells [51, 91]. From a quiescent state, fibroblasts can be reversibly or irreversibly activated to form myofibroblasts in response to different inputs (Fig. 2.1). Myofibroblasts, induced by TGF-β-mediated signaling, proliferate, gain contractile properties, and unleash an injury response to repair the cellular damage and to restore tissue homeostasis [23, 26, 66, 111]. When fibroblast activation persists even in absence of the initial injury (e.g., in chronic tissue damage or fibrosis), a pathological remodeling occurs, partly depending on epigenetic regulation [121], and tumor initiation is promoted [27, 29, 98], so that tumors are considered "wounds that do



	QF	MF	CAF
Contractility	-	++	+
Secretory phenotype	-	+	++
ECM production	-	+	+
Proliferation	-	+	++
Migration	-	+	+
Self activation	-	-	+

Fig. 2.1 Activation of fibroblasts in the tumor microenvironment, from quiescent resident fibroblasts to activated myofibroblasts to hyperactivated CAF, with sequential acquisition of key phenotypical and functional features

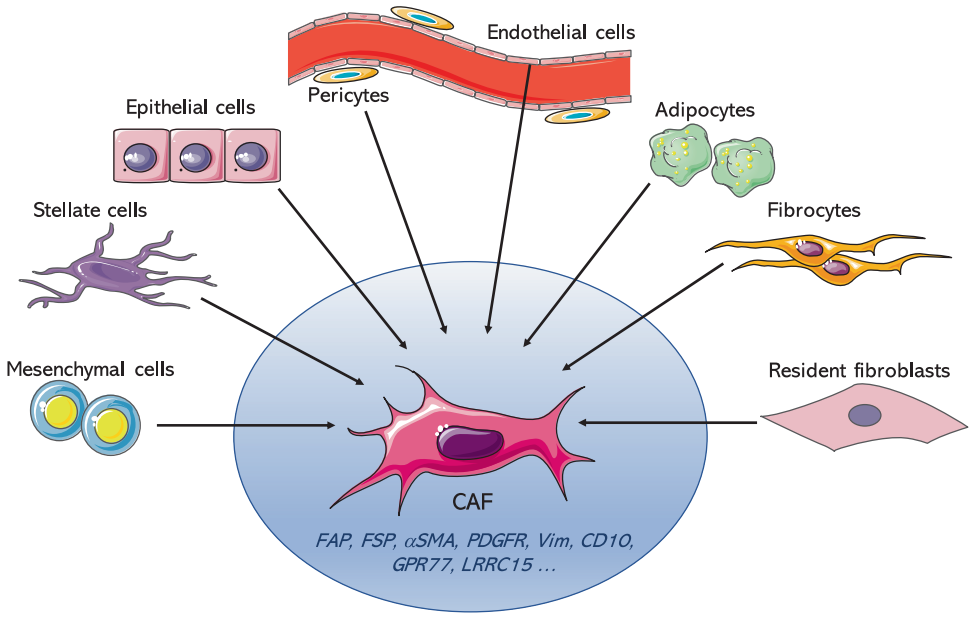


Fig. 2.2 CAF origins in the tumor microenvironment. The acquisition of a CAF phenotype is associated with the expression of a variety of CAF-related markers

not heal" [30]. By supporting tumorigenesis and by interacting with cancer cells at all tumor stages, hyperactivated fibroblasts gain enhanced proliferative properties and become a functionally diverse population, called cancer-associated fibroblasts (CAF) [52]. CAF may derive from a variety of cells, including normal fibroblasts, but also surrounding endothelial cells, pericytes, stellate cells, bone marrow-derived mesenchymal cells, and adipocytes [52, 60, 92]. Depending on their origin, the function of such activated fibroblasts can be diverse and unique (Fig. 2.2). Mediators for CAF transformation are growth factors, cytokines, and micro-RNAs soaked in the tissue *milieu* that can modulate the cellular response through a variety of molecular mechanisms [34]. In the early stages of neoplasia, the pathological tissue remodeling may initiate tumor-promoting functions in fibroblasts through the secretion of pro-inflammatory cues, such as interleukin (IL)-1β by immune cells [36]. Later, as the tumor grows, most of the CAF-transforming factors, including TGF-β, platelet-derived growth factors (PDGF), and FGF2, derive from direct secretion by cancer or stroma cells, either as soluble factors or transported by exosomes [3, 10, 34, 63, 65]. Moreover, matrix stiffness and solid stress in the TME constitute additional physical factors that cause sustained activation of CAF, through a feedback loop involving YAP activation and Rho-associated protein kinase (ROCK) signaling pathways [14].

Multifaceted bio-functions of CAF aim to orchestrate the TME and manage the tumor-stroma interface via intercellular contacts, secretion of a number of factors, modification of the ECM, and promotion of malignant transformation of epithelial cells [67, 82]. CAF contribute to hypoxia-dependent tumor neo-angiogenesis and are key actors in the restricted penetration of drugs and nanodrugs in the tumor tissue, thus modifying tumor responsiveness and therapeutic efficacy of several drugs [52, 61]. Additionally, there is evidence that CAF promote cytotoxic T cell exclusion from the tumor and hinder antitumor immune responses [57].

2.2.2 Coexisting CAF Subsets

Unlike normal fibroblasts, CAF are characterized by an increased expression of certain biomarkers, which have been recently studied as potential

targets for innovative therapeutics [16, 18, 112]. Depending on tumor type and origin, CAF express high levels of alpha-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), fibroblast specific protein 1 (FSP1 or S100A4), vimentin, and platelet-derived growth factor receptor (PDGFR)-α and β [53, 85, 86, 107, 118]. Leucine-rich repeat containing 15 (LRRC15) membrane protein, CD10, and G protein-coupled receptor 77 (GPR77) were also found highly expressed in CAF in many solid tumors [24, 59, 89, 108]. Unfortunately, none of the identified markers are currently able to select CAF with a high degree of specificity, because of a high-grade heterogeneity characterizing this cell population [2]. As an example, the loss of caveolin 1 (CAV1) expression in breast tumor cases defines fibroblasts with pro-tumorigenic functions [102]; however, a high expression of CAV1 in CAF could also facilitate tumor invasion *via* ECM remodeling [41]. Thus, nowadays, it is becoming increasingly recognized that CAF represent a heterogeneous cell population of multiple origins [49]. Researchers have demonstrated the existence of distinct subsets of CAF with different localization within the tumor mass and specificity per tumor type [79, 109]. The existence of four CAF subsets has been demonstrated in triple-negative breast cancer (S1–4) and pancreatic ductal adenocarcinoma (subsets A-D). All subtypes have unique properties and expression profiles, as assessed by marker analysis and transcriptomic investigation [6]. Of note, a specific CAF phenotype corresponds to a prognostic impact. In breast cancer, S1-CAF are associated with immunosuppressive TME by promotion of T cell differentiation into T-reg, while S4-CAF are associated with high CD8+ T cell infiltration into the tumor [21].

2.2.3 Friend or Foe?

In many tumors CAF accumulation in the TME is often correlated with poor prognosis [7, 118]. Indeed, their presence is an effective predictor of tumor reoccurrence in colorectal cancer patients and has been highlighted as a significant prognostic factor in a number of other tumor types [12, 13]. At the same time, the functional role of CAF in cancer progression and metastasis is emerging as being complex and bimodal, with both cancer-promoting and cancer-restraining actions. Recent studies have suggested that CAF can restrain pancreatic ductal adenocarcinoma (PDAC) by reducing fibrosis and hypoxia [95]. Also, patients with high desmoplasia can have improved prognosis and overall survival in PDAC, breast cancer, and lung cancer, as demonstrated by correlation studies between CAF markers and disease outcome [38, 84]. CAF have also been suggested to play a tumor-suppressive role via the I kappa B kinase/NF-kB pathway, lowering hepatocyte growth factor (HGF) secretion and reducing tumor size and metastasis [79]. Keeping all of this in mind, CAF are not a unique population, but rather an updated description of CAF requires taking into consideration their dynamic state, with epigenetic changes and variable gene expression and functions.

2.3 Fibroblasts and Tumor Progression: A Key Role in Tumor Architecture Remodeling and Desmoplasia

Over time, researchers have progressively realized that initiation, proliferation, invasion, and metastases of tumors do not rely on tumor cells properties alone, but they are influenced by the pathological stroma. From the "seed and soil" hypothesis, it has been recognized that the dynamic crosstalk between cancer cells ("seed") and TME ("soil") has a pivotal relevance in a variety of processes such as proliferation, migration, invasion, survival, angiogenesis, and EMT [83]. Through EMT, cancer cells gradually lose their epithelial hallmarks and acquire mesenchymal properties related to invasiveness and the remodeling of surrounding ECM [58]. The final result of EMT is the capability of cancer cells to reach blood circulation and metastasize at distant sites, making cancer progress from an in situ lesion to an invasive disease [46]. CAF have been

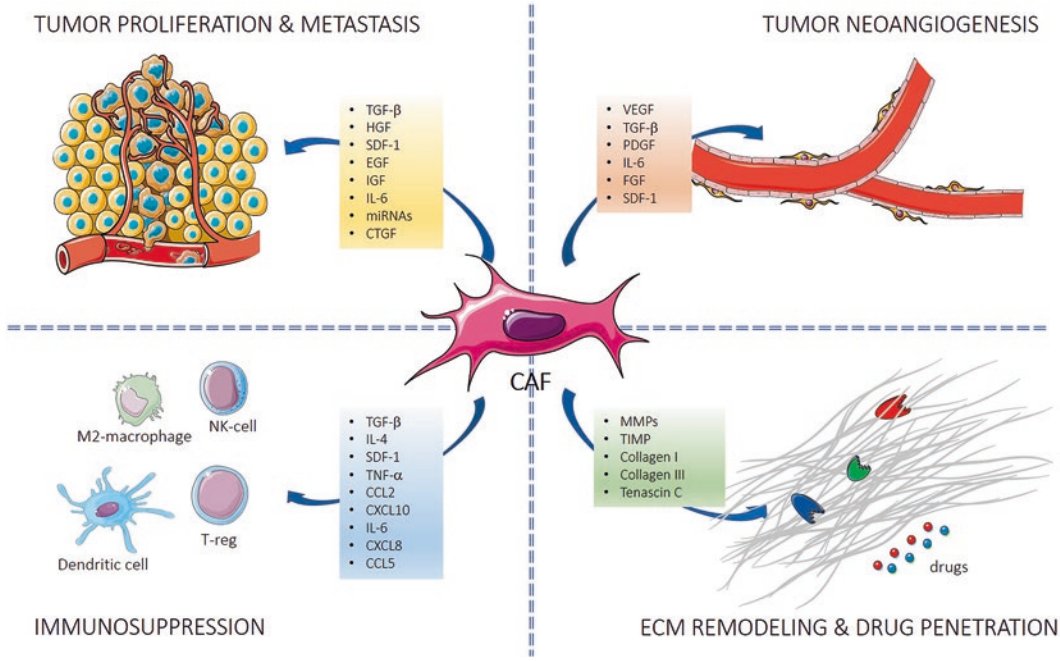


Fig. 2.3 Multivalent activity of CAF and their secretome for shaping the tumor microenvironment

shown to support cancer cell growth and metastatic dissemination in several ways [11, 51, 97] (Fig. 2.3). Their effects are mediated through both paracrine and autocrine stimulation by a variety of growth factors and cytokines, including TGF-β, bFGF, vascular endothelial growth factor (VEGF), PDGF, and interleukins (IL) [75]. TGF-β/TGF-βR signaling is required for advanced carcinogenesis via EMT induction, angiogenesis, and the modification of the stromal compartment [22, 42]. CAF-derived TGF-β1 was identified as a central molecular regulator of mesenchymal stem cells as well as a tumor-promoting factor in prostate cancer and other types of carcinoma [68, 71, 96]. Other important cues that drive the gaining of mesenchymal traits include HGF, stromal-derived factor-1α (SDF-1), osteopontin (OPN) and key cytokines released by CAF able to reprogram cancer cells through activation of the Wnt/β-catenin signaling pathway, which fosters migration and metastasis [110]. HGF and IL-6 are also considered drivers of tumor initiation and progression, through their interaction with MAPK, PI3K/Akt, and JAK/STAT signaling pathways, along with the subsequent induction of c-MET expression as positive feedback regulation [25, 116]. The coordination of these pathways controls tumorigenic progression in response to CAF's paracrine activity. CAF-derived SDF-1, also known as CXCL12, is also able to induce an angiogenic response in synergy with the chemokine ligand CXCL -8 and enhances the invasiveness of pancreatic cancer cells [69, 81]. A gene knockdown assay and gain- and loss-of-function assays revealed that CAF secrete TGF-β and SDF-1, which promote the formation of capillary-like structures, participate in vascular endothelial cells migration, tube formation, and angiogenesis via interaction with TGF-βR1 and CXCR-4 in tumor cells [37, 119].

The ability to control the local remodeling of ECM is another critical function of CAF and a feature of paramount importance during the desmoplastic reaction occurring in many carcinomas. Activated fibroblasts are an important source of ECM-degrading proteases, including matrix metalloproteinases (MMP), zinc-dependent endopeptidases that facilitate cancer cell migration across ECM [101, 105, 114]. MMP-3, produced by CAF, promotes EMT by

cleavage of E-cadherin and induces invasiveness of cancer cells [64, 104]. MMP-13 promotes angiogenesis by releasing VEGF and increasing the invasive capabilities of squamous cell carcinoma cells [56]. Additionally, other stromal MMP, such as MMP-1, MMP-9, and MMP-14, are able to induce cancer invasiveness, and their expression has been associated with tumor progression in several carcinomas [8, 106].

2.4 Cancer-Associated Fibroblasts in Immunosuppression and Chemoresistance

Generally, CAF are known to promote an immunosuppressive TME. Fibroblasts are a significant source of immunomodulatory cytokines and chemokines, notably interferon-γ, IL-6, CCL2, and tumor-necrosis factor-α, which can influence the mobilization of cytotoxic T lymphocytes, natural killer cells, and macrophages [43, 93, 100] (Fig. 2.3). Paracrine CAF–immune cell signaling may induce differentiation of immunosuppressive myeloid cells and affect macrophage recruitment to the tumor [55, 72, 113]. T cell recruitment and activation also involves cytokines that are found in the CAF secretome, such as CXCL9, CXCL10, and SDF-1 [5]. A recent study has shown that programmed cell death 1 ligand (PDL) 1 and 2 in a subset of CAF derived from patients with lung cancer may carry an immunosuppressive effect on T cell activation ex vivo [77, 87].

Beyond CAF secretome which switches off anticancer immunity, CAF-orchestrated ECM plays a crucial role in restricting access of immune cells to cancer, by generating a physical barrier to tumor infiltration and unmasking cryptic binding sites that could promote immune cell adhesion [33, 50]. In orthotopic tumor grafts, targeting FAP+ CAF with a DNA vaccine showed antitumor effects via suppression of collagen synthesis and intratumoral recruitment of CD8+ T cells, with the subsequent immuno-control of tumor growth [62, 80]. CAF distribution at the interface between blood vessels and tumor cells contributes to increasing the tumor interstitial fluid pressure, which represents a physical barrier to several drugs [7]. Moreover, dynamic ECM alterations may induce tissue stiffening and increased tension, which have been associated with poor outcome in patients with many solid tumors [14]. The immunosuppressive and poorly accessible TME drastically limits the potential of effective therapeutics, which have raised new hopes for the treatment of several malignant tumors. Therefore, favoring ECM remodeling and overcoming immunosuppression in the tumor is of fundamental importance for effective anticancer treatment.

2.5 Targeting Cancer-Associated Fibroblasts: Current Clinical Evidence

Considering the central role of CAF in cancer progression and diffusion, it is quite surprising that TME-targeted treatments have been so poorly explored in clinical trials to date. A main reason for the lack in clinical data is the relatively difficult specific targeting of CAF. A promising candidate for CAF targeting is FAP, a cell surface glycoprotein expressed in over 90% of these stromal cells while normally not expressed in most healthy tissues. In 1994 a first phase I study evaluated the clinical use of a monoclonal antibody toward FAP for imaging purposes, labeling it with iodine 131, to detect liver metastases from colorectal cancer [115]. In accrued patients, iodine 131-labeled anti-FAP antibodies were administered 1 week before liver surgery or regional chemotherapy, demonstrating a high accumulation within liver metastases but not in liver normal parenchyma, and no significant toxicities. Therefore, a first proof-of-concept on selective overexpression of FAP in metastatic colorectal cancer was provided, together with the usefulness of focusing on TME for clinical purposes. Subsequently, the anti-FAP antibody named sibrotuzumab was clinically assessed for anticancer efficacy in further trials. First, a phase I clinical study evaluated sibrotruzumab in FAP+ metastatic colorectal and non-small cell lung

cancer [99]. After 12 weeks, treatment with sibrotuzumab showed no significant toxicities and was overall well tolerated. However, on the other hand, cancer progression was observed in all included patients, and no objective tumor response was reported. In another phase II trial, sibrotuzumab was administered in metastatic colorectal cancer patients: unfortunately, all patients still experienced cancer progression except for 2 cases, where a stable disease was observed [44]. Despite, yet again, the fact that no significant toxicities were reported, the trial failed to provide a benefit from sibrotuzumab and it was terminated. Furthermore, although no severe adverse events were reported, it should be noted that FAP is overexpressed also in bone marrow, further making clinical translation difficult. The discouraging findings from the above-mentioned trials have resulted into a long-lasting abandonment of the interest toward sibrotuzumab,; however it has also produced a number of lines of thought. Targeting the TME could probably be a winning strategy in preventing reactivation and progression of dormant metastatic tumor cells, rather than arresting the metastatic storm once the TME has elicited its promoting activity [19]. Indeed, once cancer progression has started and metastatic disease occurs, a large amount of cancer-promoting forces are activated, making it difficult to be effectively counteracted by targeting tumor stroma only. Targeted therapy for TME might therefore be preferred as an ancillary treatment to support conventional chemotherapy in the first-line therapy of cancer, since its anticancer efficacy as a stand-alone treatment is limited, as demonstrated in preclinical studies on FAP inhibition [20] or in clinical trials targeting other TME actors, such as metalloproteinases [78]. More recently, another approach to target CAF activity has been proposed, based on inhibition of FAP enzymatic activity rather than targeting FAP itself. In a phase II clinical trial, Talabostat, an orally available amino boronic dipeptide which competitively inhibits the dipeptidyl peptidase activity, has been administered as a stand-alone therapy in metastatic colorectal patients previously treated with conventional chemotherapy [76]. Although 21% of patients maintained a stable disease for up to 25 weeks, no objective responses were observed, demonstrating a minimal clinical activity of Talabostat. However, since it was tolerated well by patients, Talabostat was further assessed in non-small cell lung cancer patients in combination with docetaxel; however, only 3 patients out of 42 reported an objective response [31]. Since Talabostat has been related to increased production of cytokines leading to enhanced antitumor immunity [1], this FAP inhibitor represented a hope for new treatment approaches in highly immunogenic malignancies, such as melanoma. Inspired by the intriguing discovery that Talabostat with cisplatin makes mice resistant to rechallenge with melanoma cells, a phase II trial evaluating Talabostat and cisplatin as a second-line therapy for metastatic melanoma was conducted [32]. A partial response was observed in less than 10% of included patients, similarly to treatment with cisplatin alone: thus, Talabostat added no clinical benefit. Furthermore, about one-third of patients experienced severe side effects related to the use of Talabostat, mainly anemia, thrombocytopenia, and neutrophilia. Regarding the minimal clinical effect, it should be noted that Talabostat acts by inhibiting the peptidase activity of FAP only; however, it was recently demonstrated that FAP promotes cancer growth and progression also through non-enzymatic activities, such as stimulating ECM remodeling by MMP-9 [47]. Phase III clinical trials on Talabostat combined with docetaxel or pemetrexed for treatment of late-stage non-small cell lung cancer were initiated, but these studies were prematurely stopped at the interim evaluation due to the observation of a lower survival rate in the Talabostat group compared to the placebo group [9]. The current difficulty in targeting FAP or in inhibiting its enzymatic activity has not decreased the great interest in implementing an effective strategy toward TME in cancer management. Indeed, while the targeting of cancer cells must follow their evolving wide heterogeneity with frequent onset of resistance, TME and interactions between cancer and TME are much more universal and common to different types of cancer,

making targeting TME a promising approach. Therefore, an innovative strategy was proposed which focuses on growth factors deriving from CAF, such as FGF. Nintedanib is a pan-tyrosin kinase inhibitor, acting toward receptors for FGF, VEGF, or PDGF, overexpressed in cancer cells. By inhibiting the activity of the above-mentioned growth factors, the downstream support from CAF to cancer cells could theoretically be reduced or abolished, avoiding stimulation of tumor proliferation, migration, and survival. In 2010 Nintedanib was evaluated in a phase I clinical trial on 61 patients affected by advanced solid malignancies, demonstrating to be limited by G3-G4 reversible liver enzyme elevation but substantially showing a decent level of tolerability on behalf of patients. Despite the fact that only 3 clinical responses were reported, in 55% of patients, a significant reduction in tumor blood flow was observed, suggesting that targeting a CAF-derived growth factor may significantly impact on TME and its neoangiogenesis [74]. A further clinical trial of Nintedanib in advanced or metastatic relapsed non-small cell lung cancer administration achieved disease stabilization in 46% of patients, with a median progression-free survival of 6.9 weeks [94]. These encouraging findings warranted further clinical exploration of this strategy, and after the finding that Nintedanib in addition to docetaxel improves the overall survival rate, it is currently an established second-line treatment for non-small cell lung cancer [88]. Under the new perspective of targeting the signaling network of CAF, a monoclonal antibody toward TGF-β has been developed and named Fresolimumab. Recent clinical trials have evaluated Fresolimumab in previously treated melanoma, renal cell cancer [73], or metastatic breast cancer [39], but a limited clinical response was conjugated with the occurrence of secondary cutaneous malignancies, stopping any further clinical trial with TGF-β antagonists. Indeed, TGF-β may stimulate cancer in advanced stages making its inhibition a potential anticancer treatment; on the other hand TGF-β could mediate inhibition of cancer development in normal tissues [35].

2.6 Future Trends for Cancer Therapy Through Fibroblasts

2.6.1 CAF Reprograming

As suggested by the major concerns emerged from *tout-court* CAF-inhibiting strategies, TME might play several different roles in cancer progression, including both cancer-promoting and cancer-suppressing pathways. TME was classically depicted as a stable and universal feature of cancer, while it is increasingly recognized that it is highly heterogeneous. The coexistence of different subpopulations of CAF has been proposed, ranging from cancer-inhibiting to cancer-enhancing fibroblasts [51]. Therefore, a precision medicine approach should also be preferred in targeting CAF, and turning CAF from a cancer-enhancing profile to one that is cancer-inhibiting might be a more suitable strategy than the total depletion of CAF. Two recently proposed specific surface biomarkers of tumor-enhancing CAF are CD10 and GPR77, and a monoclonal antibody toward the latter receptor has shown reduced chemoresistance in a patient-derived breast cancer xenograft [108]. Beyond a precise targeting of cancer-supporting CAF, the main challenge is how to reprogram them in order to convert an immunosuppressive into an immune-permissive TME. An interesting approach has been proposed to block those signals fueling fibroblast activity, such as the angiotensin II-angiotensin II receptor type-1 axis. Indeed, angiotensin II transforms quiescent fibroblasts into CAF; therefore angiotensin receptor blockers (ARBs) should hypothetically reverse the process and reprogram CAF. A clinical concern is represented by the potent antihypertensive effects of ARBs, making them useless as anticancer treatment in clinical practice. However, ARBs have been recently nano-conjugated with pH-dependent degradable polymers in order to selectively direct ARBs into the acidic TME in a murine model of metastatic breast cancer [15]. Intriguingly, this strategy allowed for the reprogramming of CAF without hypotensive effects, deleting the immunosuppression promoted by TME and improving the

T lymphocyte antitumor response, thus extending survival of mice with concurrent administration of immune checkpoint blockers. Another original approach for CAF reprograming is based on epigenetic regulation. The use of a selective inhibitor of histone deacetylases (HDACs) has been successfully used to interfere with TGF-β-mediated CAF differentiation, thus reversing CAF activation and delaying cancer growth [54].

2.6.2 Immunotherapy

Following the increasing interest toward antitumor immunity and strategies based on enhancement of T cell responses to cancer cells, a similar approach may be translated as anti-CAF treatment. In particular, combined treatments toward cancer cells and CAF are particularly promising. A specifically engineered T-cell engager for both FAP and human CD3 has been inserted into an oncolytic virus: the binding with CD3+ effector T lymphocytes and with FAP-expressing CAF lead to T cells activation and cytotoxicity toward CAF, while the oncolytic activity of the viral vector exerted its well-known anticancer effect [103]. This oncolytic approach not only results in CAF depletion, but it may also mediate a reversal of TME from immunosuppressive to immune-permissive, as shown by the repolarization of M2 macrophages toward a proinflammatory profile [40] in fresh prostate cancer tissue derived from biopsy samples. In other words, tumor-infiltrating lymphocytes could be reeducated to kill CAF, leading to TME remodeling and cancer suppression. Beyond oncolytic viruses, an elegant solution for priming the natural intratumoral immune response toward CAF is the use of specific vaccines. Tolerance toward FAP can be broken by specific DNA vaccines to exploit the cytotoxic activity of CD8+ and CD4+ T lymphocytes toward CAF. Interestingly, the T cell-mediated CAF depletion also decreased macrophage infiltration and increased intratumoral lymphocytes; furthermore this strategy was improved by adding tumor-specific DNA vaccines in different cancer models [28]. As previously stated, targeting TME as a stand-alone therapy might be ineffective, especially in aggressive cancers or where metastatic spread has already occurred. A combination strategy toward both cancer and TME could maximize the outcome. Therefore, other DNA vaccines to prime cytotoxic T lymphocytes toward FAP-positive CAF have been developed and tested in combination with chemotherapeutics with immunomodulatory activity, such as cyclophosphamide [117], demonstrating enhanced anticancer efficacy. An original sort of FAP-specific vaccination has been proposed by fusing dendritic cells, which normally present antigens to start the immune response, with CAF [90]. The resulting hybrid cells effectively activated T cells to generate a specific cytotoxic immune response toward CAF, inhibiting cancer growth.

2.6.3 Nano-strategies to Target CAF

Nanoparticles have been profoundly explored as an excellent drug delivery system in tumors, first exploiting their natural intratumoral delivery due to extravasation from leaky vasculature (the so-called enhanced permeability and retention effect, EPR). Then, by conjugation with specific antibodies, nanoparticles have been increasingly evaluated for actively targeting cancer. In both cases a high anticancer efficacy combined with a significantly lower toxicity have been reported, thanks to the specific action of drugs loaded inside cancer cells, thus avoiding off-target adverse effects in healthy tissues. Despite nanomedicine demonstrating great potential for cancer treatment, its clinical translation is a slow process, due to production costs and safety concerns. A special interest in nanomedicine has recently been developed also for targeting TME. Nano-liposomes conjugated with a peptide recognizing tenascin C, overexpressed in CAF, have been demonstrated to adequately address the anti-apoptotic drug Navitoclax in TME [17]. As a consequence, downregulation of ECM deposition, decreased interstitial fluid pressure, and increased blood perfusion with a subsequent improvement in chemotherapeutics penetration

have been observed. The reduction in the high intratumoral interstitial pressure due to TME has been observed also with gold nanoparticles in xenograft of colorectal cancer [123]. Interestingly, after treatment with naked gold nanoparticles, CAF and pro-fibrotic signals decreased as well as TME stiffness, leading to increased penetrance and activity of cisplatin, which was subsequently administered. Similar findings were reported also for ovarian cancer, where gold nanoparticles were demonstrated to affect the VEGF signaling, thus blocking neoangiogenesis by disrupting the cancer cell-TME crosstalk [122]. The innate capability of untargeted gold nanoparticles to inhibit the interaction between cancer cells and TME has been more deeply studied: not only do they act on AKT pathways and VEGF signaling, they also modulate cancer cell secretome to reduce the desmoplastic feature in pancreatic cancers [70]. A more intriguing feature of gold nanoparticles might explain their natural anti-TME effects not only affecting cell crosstalk but also finely modulating the CAF profile. As recently demonstrated, gold nanoparticles increase lipid intracellular content by inducing an expression of lipogenesis genes in CAF, which use endogenously synthetized lipids to convert into quiescent fibroblasts [45]. Also, actively targeted nanoparticles toward CAF have been evaluated. A biocompatible ferritin-based nanocage has been engineered with a FAP-specific single-chain variable fragment to provide a prompt targeting and internalization into CAF, for subsequent photoirradiation exploiting the photosensitizing feature of ferritin [124]. By this nano-based photoimmunotherapy, CAF were efficiently depleted, enhancing T cell infiltration and tumor suppression in immunocompetent mice, again providing a proof-of-concept on the usefulness of targeting TME to increase antitumor immunity.

An increased interest toward implementation of anti-TME treatments for cancer therapy is expected over the next years. After the failure of clinical trials to demonstrate a significant benefit provided by anti-FAP monoclonal antibodies, it appeared clearer that, beyond merely killing CAF, other strategies aiming at reeducating CAF to modulate TME merit further exploration. Promising therapies in reaching this goal are selective inhibitors of CAF signaling, DNA vaccines toward CAF, and targeted nanodrugs; however, further characterization of CAF molecular biomarkers is needed in order to exploit suitable targets and thus avoid a tout-court action on all fibroblasts, including those providing anti-cancer activity, and avoid off-target toxicities. Finally, a selective modulation of TME could be an optimal treatment to prevent the invasive features of primary cancer or, in the best case, to prevent metastatic cancer cells in distant niches, but its potential efficacy for advanced/metastatic cancers is much less clear and combination strategies with cytotoxic drugs could maximize the outcome in these cases.

Acknowledgment The authors thank Associazione Italiana per la Ricerca sul Cancro for research support (AIRC IG 20172 to F.C.)

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Mesenchymal Stem Cells in the Tumor Microenvironment

3

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Abstract

The interactions between tumor cells and the non-malignant stromal and immune cells that make up the tumor microenvironment (TME) are critical to the pathophysiology of cancer. Mesenchymal stem cells (MSCs) are multipotent stromal stem cells found within most cancers and play a critical role influencing the formation and function of the TME. MSCs have been reported to support tumor growth through a variety of mechanisms including (i) differentiation into other pro-tumorigenic stromal components, (ii) suppression of the immune response, (iii) promotion of angiogenesis, (iv) enhancement of an epithelial-mesenchymal transition (EMT), (v) enrichment of cancer stem-like cells (CSC), (vi) increase in tumor cell survival, and (vii) promotion of tumor metastasis. In contrast, MSCs have also been reported to have antitumorigenic functions including (i) enhancement of the immune response, (ii) inhibition of angiogenesis, (iii) regulation of cellular signaling, and (iv) induction of tumor cell apoptosis. Although literature supporting both arguments exists, most studies point to MSCs acting in a cancer supporting role within the confines of the TME. Tumor-suppressive effects are observed when MSCs are used in higher ratios to tumor cells. Additionally, MSC function appears to be tissue type dependent and may rely on cancer education to reprogram a naïve MSC with antitumor effects into a cancer-educated or cancer-associated MSC (CA-MSC) which develops pro-tumorigenic function. Further work is required to delineate the complex crosstalk between MSCs and other components of the TME to accurately assess the impact of MSCs on cancer initiation, growth, and spread.

Keywords

Mesenchymal stem cells · Tumor microenvironment · Cancer · Immune response · Angiogenesis · Cancer stem cell · Stroma · Epithelial-mesenchymal transition

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_3

3.1 Introduction

In the course of neoplasia, tumor cells (TCs) extensively interact with adjacent cell populations in the "tumor microenvironment (TME)." The TME is a complex network of non-malignant stromal and immune cells which surround the cancerous tissue. Interactions with microenvironment cells cause TCs to undergo genetic and functional changes that increase metastasis, enhance proliferation, and induce chemotherapeutic resistance [1, 2]. In addition, the TME also contains a non-cellular component consisting of the extracellular matrix (ECM) and soluble factors. Studies have shown that ECM and soluble factors in the TME play an important role in supporting tumor progression, and these factors are strongly associated with tumorigenesis [3].

Mesenchymal stem cells (MSCs) (also known as multipotent mesenchymal stromal cells) are non-hematopoietic multipotent stromal stem cells that can be found in a variety of tissues, such as ovary, brain, spleen, liver, kidney, lung, muscle, thymus, pancreas, adipose, and bone marrow. MSCs are distinct from other stromal cells, such as fibroblasts, and MSCs have a unique expression profile that is positive for stromal cell markers (CD73, 105, 44, 29, and 90) but negative for endothelial (CD34, 31, and vWF) and hematopoietic (CD45 and 14) markers [4]. MSCs are progenitor cells to multiple stromal components, possessing the ability to differentiate into osteocytes (bone), adipocytes (adipose), chondrocytes (cartilage), and fibroblasts [5]. Given the lack of one specific marker and the fact that they are closely related to more terminally differentiated stromal cells, the identification of MSCs can be challenging. The international society for cellular therapy published minimal criteria for defining multipotent mesenchymal stroma cells which state MSCs (1) must be plastic adherent; (2) must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA-DR surface molecules; and (3) must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [4] (Fig. 3.1).

Scholarly literature presents divergent evidence on the role of MSCs in the TME and cancer progression. Both pro-tumorigenic and antitumorigenic functions have been ascribed to MSCs; this dichotomous relationship can be attributed to the heterogeneity in MSC definition, source of MSC derivation, and methods of study. Thus, this chapter will present evidence of the pro- and antitumorigenic functions of MSCs and will discuss potential reasons for the existence of this apparent contradiction.

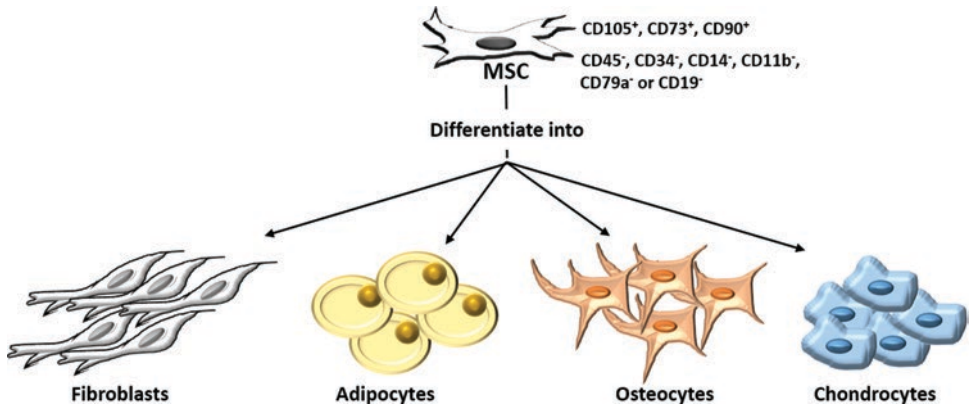

Fig. 3.1 The definition of mesenchymal stem cell (MSC). MSCs express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14, CD11b, CD79a, or CD19. MSCs also differentiate to fibroblasts, osteoblasts, adipocytes, and chondrocytes in vitro

3.2 Pro-tumorigenic Function of MSCs in the TME

Within the confines of the tumor microenvironment, tumor-secreted factors and direct TC-MSC interactions induce a pro-tumorigenic phenotype in the MSC population, creating carcinoma-associated mesenchymal stem cells (CA-MSCs) [6, 7]. CA-MSCs retain their differentiation capacity and stromal surface markers, but they contribute to tumor progression via several mechanisms: (i) differentiation into other pro-tumorigenic components of the TME, (ii) suppression of immune response, (iii) promotion of angiogenesis, (iv) enhancement of an epithelial-mesenchymal transition (EMT), (v) enrichment of cancer stem-like cells (CSC), (vi) increase in tumor cell survival, and (vii) promotion of tumor metastasis [6, 8–13].

3.2.1 Differentiation into Pro-tumorigenic Components of the TME

A defining characteristic of MSCs is their ability to differentiate into multiple cell lineages, such as fibroblasts, adipocytes, osteocytes, and chondrocytes. These multipotent properties suggest that MSCs may play a key role in the generation of most stromal components of the TME. Multiple reports have demonstrated that CA-MSCs differentiate into tumor supporting carcinoma-associated fibroblasts (CAFs) and adipocytes (CAAs) in the presence of tumor cells.

3.2.1.1 Carcinoma-Associated Fibroblasts

The traditional role of fibroblasts is to facilitate wound healing by regulating extracellular matrix remodeling [14]. Within the confines of the TME, CAFs constitute the majority of the local stroma and contribute significantly to tumorigenesis [15]. While CAFs can be derived from local stromal fibroblasts, both resident and distally recruited MSCs have been shown to acquire a CAF-like phenotype within the TME niche [16]. Interestingly, CA-MSCs demonstrate an even greater ability to differentiate into CAFs versus normal MSCs within the TME [17]. While the exact mechanism underlying CA-MSC to CAF differentiation has not yet been elucidated, there is growing evidence that tumor-secreted factors induce the TGF-β/Smad signaling pathway in MSCs drive differentiation into a CAF phenotype [18, 19]. Additionally, the CAF phenotype is stable and persists in in vitro cell culture sans tumor stimulation [20].

Pro-tumorigenic functions of CAFs include increased tumor cell invasion, enhanced EMT through Hedgehog signaling, ECM remodeling resulting in increased desmoplasia, promotion of tumor initiation in pre-malignant cells, increased CSC profile, promotion of migration and metastasis, and increased chemotherapeutic resistance [21–27].

3.2.1.2 Carcinoma-Associated Adipocytes

Adipocytes are a major component of adipose tissue, and they function in both lipid storage and signaling regulation. Adipocytes generate a variety of growth factors, hormones, cytokines, and adipokines. Specifically, CAAs have a unique secretome that aids in extracellular matrix remodeling, invasion, therapeutic resistance, and EMT [28]. Increased insulin-like growth factor binding protein-2 (IGFBP-2) expression and secretion in CAAs was shown to enhance migration and invasion in in vitro and in vivo breast cancer models [29]. Additionally, co-culture of ovarian cancer cells and CAAs exhibited enhanced migration and invasion of the cancer cells through increased production and secretion of IL-8/fatty acid binding protein-4 [30].

3.2.2 Suppression of Immune Response

Canonically, MSCs play a role in healing damaged tissues, engaging in direct and paracrine crosstalk with immune cells [31]. MSCs demonstrate chemotaxis towards inflammatory chemo-

kines released by damaged tissues, migrating to the wound and suppressing both innate and adaptive immunes responses [32]. Dendritic cell (DC) differentiation is suppressed when MSCs downregulate interferon-γ (IFN-γ) and TNF-α expression [33]. Direct cell-to-cell interactions between MSCs and natural killer (NK) cells alter the phenotype of NK cells, suppressing proliferation and cytokine secretion [34]. Macrophages co-cultured with MSCs favor M2 polarization, leading to an increase in phagocytic activity and decreased expression of inflammatory cytokines IFNγ, TNF-α, IL-1β, and IL-12 [35, 36]. Additionally, soluble factors secreted by MSCs have been shown to repress T- and B-cell proliferation while increasing apoptosis in activated T cells [37–39].

In the context of the TME, CA-MSCs use similar mechanisms to support tumor growth. Mounting evidence suggests that CA-MSCs can regulate the proliferation and maturation of DCs, NK cells, T cells, and B cells [34, 40–42]. Additionally, CA-MSCs promote immunosuppression by secreting the cytokines IL-10, TGFβ, nitric acid, indoleamine 2,3-dioxygenase, and prostaglandin E2 [43, 44]. In vivo studies using murine melanoma tumor models have shown that IFN-γ and TNF-α promote the immunosuppressive role of CA-MSCs, enabling increased tumor growth [11, 45]. A mouse model of pancreatic cancer likewise demonstrated CA-MSCs promote cancer growth through M2 macrophage polarization [46]. Another study using a prostate cancer model demonstrated that MSCs significantly increase tumor initiation and growth through suppression of the immune response [47].

3.2.3 Promotion of Angiogenesis

The induction of angiogenesis is a hallmark of cancer and is considered one of the early steps in the development of invasive cancers [48]. Angiogenesis is the development of new blood vessels from existing vasculature and is necessary to sustain expanding tumor growth. An increasing amount of evidence suggests that angiogenesis is governed by MSCs within the TME. Work in syngeneic mouse models has shown that co-injection of MSCs supports the formation of tumor neo-vasculature by localizing close to the vascular walls and by expressing CD31 [10]. There is also evidence that MSCs secrete a number of soluble pro-angiogenic factors, such as LIF, M-CSF, MIP-2, VEGF, IFN-γ, and TNFα. Moreover, MSCs can enhance angiogenesis through induction of the ERK1/2 and p38 MAPK pathways, which enhance the expression of VEFG and CXCR4 in tumor cells [49]. CA-MSCs, via a paracrine signaling loop involving BMP4 and Hedgehog, also induce angiogenesis in ovarian cancer models [13]. Collectively, this research suggests that CA-MSCs appear to play a role in tumorigenesis via promotion of neovascularization.

3.2.4 Enhancement of the Epithelial-Mesenchymal Transition (EMT)

The detachment of cancer cells from the primary tumor, otherwise known as dissemination, is the initial step in metastatic spread. Dissemination is found to be tightly associated with the epithelial-mesenchymal transition (EMT), a process in which epithelial cells undergo multiple changes to gain mesenchymal properties. EMT is typically an embryonic process. However, increasing evidence shows that the TME stimulates EMT in cancer cells through the activation of the same pathways stimulated during embryogenesis. Both embryonic and cancerous EMT are characterized by loss of E-cadherin, which often results from change-of-function mutations in the *CDH1* gene or from decreased E-cadherin expression. This altered expression affects downstream steps, such as the activation of transcriptional factors Snail, Slug, Twist, and FOXC2 [50]. In addition, the disruption of E-cadherin is associated with expression of N-cadherin, or mesenchymal cadherin, which facilitates motility and migration of cancer cells within the surrounding stroma [51]. MSCs can stimulate EMT in cancer cells through CCL5

production. CCL5 promotes the secretion of matrix metalloproteinase (MMPs) which act by breaking down the extracellular matrix (ECM), thereby increasing the motility of cancer cells and enhancing their metastatic ability [52]. In a pancreatic cancer model, MSCs stimulated EMT through a Notch-dependent mechanism [53].

3.2.5 Enrichment of Cancer Cell Stemness

Cancer stem-like cells (CSCs), also known as tumor initiating cells, are a subpopulation of cancer cells with the ability to recapitulate the entire tumor population and are the cells thought to be responsible for cancer initiation, chemotherapy resistance, and metastasis. A growing body of work demonstrates that MSCs enhance CSC proliferation and invasiveness via multiple pathways and in a variety of cancer types. Secretion of IL-6 by MSCs increases JAK2/STAT3 pathway activation in cancer cells, which has been shown to enhance sphere formation and tumor initiation in lung cancer [54]. Following MSC co-culture, breast cancer cells exhibit upregulated CXCL7 and IL-6 pathways and demonstrate enhanced mammosphere formation and increased self-renewal capacity [55]. In another breast cancer study, MSCs were linked to the promotion of stem cell proliferation via P2X-mediated purinergic signaling [56]. Furthermore, activation of the WNT and TGF-β signaling pathways in gastric cancer resulted in an increase of the CSC population [57]. A Hedgehog/BMP4 signaling loop between CA-MSCS and ovarian cancer cells likewise increases ovarian CSCs [13]. Taken together, these data suggest that MSCs play a significant role in enriching the CSC population and driving disease initiation, resistance, and progression.

3.2.6 Increasing Tumor Cell Survival

MSCs contribute to tumor cell survival in several ways. Within the TME, tumor progression is accompanied by hypoxia and energy starvation. Within these otherwise treacherous conditions, it has been reported that MSCs increase their cellular proliferation and stemness through the expression of Rex-1 and Oct-4 [58]. MSCs have also been shown to release many soluble factors that promote tumor survival and proliferation including VEGF, FGF-2, PDGF, HGF, brain-derived neurotropic factor (BDNF), SDF-1α, IGF-1, IGF-2, TGF-β, and IGFBP-2 [59–61]. Many of these molecules, namely, VEGF and FGF-2, mediate the expression of anti-apoptotic factor Bcl-2 in order to promote tumor cell survival [62, 63]. A study by Burger et al. demonstrated that SDF-1α expressed by MSCs can prevent drug-induced apoptosis of chronic lymphocytic leukemia (CLL) cells [64]. Another study showed that direct cell-to-cell contact with MSCs significantly enhances the viability and proliferation of glioblastoma [65]. Thus, MSCs appear to make a noteworthy contribution to the survival of tumor cells.

3.2.7 Promotion of Tumor Metastasis

During metastasis, cancer cells escape the primary tumor and eventually lead to the formation of secondary tumors in distant parts of the body. In order for primary tumors to form secondary tumors, cancer cells need to go through the sequential events of invasion, intravasation, extravasation, and colonization [66]. The process of invasion starts once cancer cells break away from the primary tumor mass. The detached cancer cells invade the basement membrane and migrate through the surrounding stroma to reach nearby blood vessels. Cancer cells then intravasate as they penetrate the lymphatic or vascular wall and travel through the circulatory system. The traveling cancer cells extravasate from the vasculature by exiting through the vascular wall and implanting into distant organs. Ultimately, the cancer cells proliferate and form tumors in their new location via a process known as colonization. The successful completion of the metastatic process is

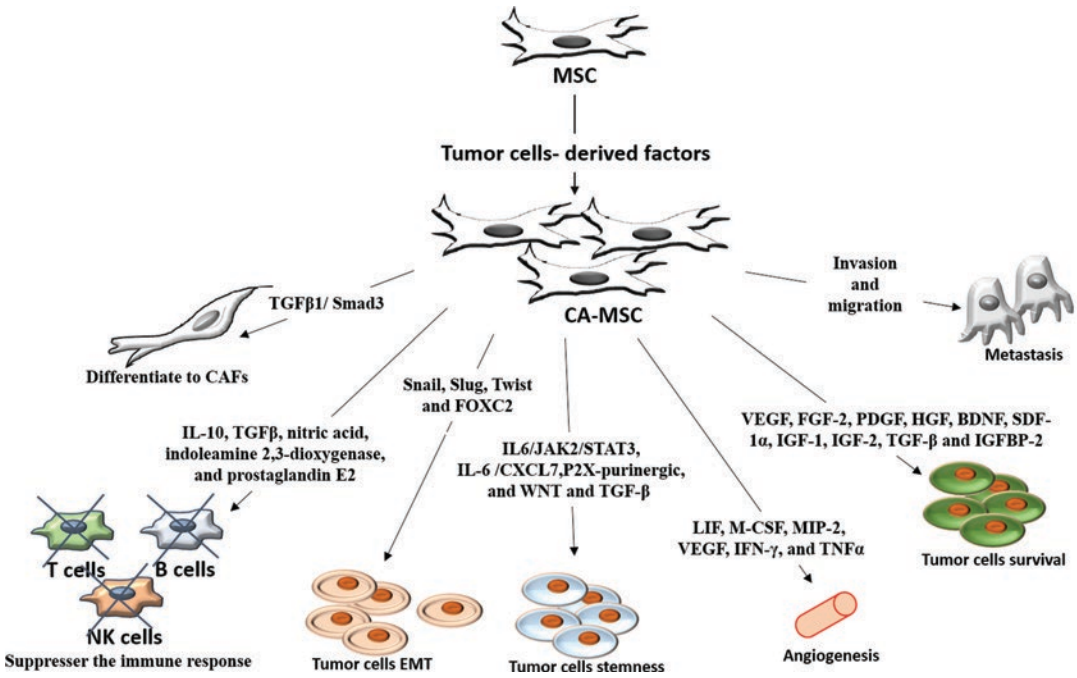

Fig. 3.2 The role of MSCs in supporting tumor progression. MSCs (1) differentiate to form cancer-associated fibroblasts (CAFs), (2) dampen the anti-tumor immune response, and (3) induce cancer cell EMT, cancer cell stemness, angiogenesis, cancer cell survival, and metastasis

determined by the ability of cancer cells to colonize distant organs [48, 67].

Studies have shown that MSCs play a crucial role in promoting metastasis through multiple mechanisms. It has been reported that MSCs secrete TGF-β which increases cancer cells' invasive and migratory potential [65]. In the breast cancer cell line MCF7, cancer cells exhibited an enhanced migratory capacity after MSC-exosome treatment, specifically through induction of the WNT pathway. Exosome treatment led to an increase in the expression of WNT target genes Axin2 and Dkk1, as well as β-catenin [68]. A different investigation identified that MCF-7 breast cancer cells have increased migration potential when co-cultured with MSCs in vitro which is mediated through ER-SDF-1/CXCR4 crosstalk [69]. It has also been reported that bone marrow-derived MSCs enhance the migratory capacity of breast cancer cell lines through the CXCR2 receptor [12]. Finally, as discussed above, MSCs promote cancer cell metastasis through inducing EMT and enrichment of CSCs [53] (Fig. 3.2).

3.3 Antitumorigenic Function of MSCs in the TME

As previously mentioned, significant controversy exists regarding the role of MSCs in cancer. In addition to the pro-tumorigenic effects described above, other studies have shown that MSCs act in an ant-tumorigenic manner to suppress disease progression. Studies both in vivo and in vitro have shown that MSCs can inhibit tumor growth and metastasis through several mechanisms such as (i) modulation of immune responses, (ii) inhibition of angiogenesis, (iii) regulation of cellular signaling, and (iv) induction of apoptosis.

3.3.1 Modulation of Immune Responses

Although MSCs have been mainly shown to suppress immune responses, there are reports of MSCs inducing an antitumorigenic immune

response. In a rat colon cancer model, MSCs inhibited cancer growth by increasing monocyte and granulocyte infiltration in the TME [70]. Further, Toll-like receptor 3 (TLR3)-activated MSCs enhance neutrophil function, and MSCs have been reported to stimulate resting T cells and act as antigen-presenting cells; however it is unclear if this happens within the TME [71, 72]. MSCs may also play a role in recruitment of different immune populations into the TME altering the ratio of Treg and myeloid-derived suppressor cells to CD8+ T cells shifting the balance towards an antitumorigenic state [73]. Interestingly, this change in immune infiltration was associated only with MSCs injected distant from the tumor rather than co-injected with tumor cells indicating naïve or non-tumor-associated MSCs may have divergent functions compared to MSCs in direct association with tumor cells.

3.3.2 Inhibition of Angiogenesis

While the pro-angiogenic functions of MSCs have been well described, there is evidence that MSCs can inhibit angiogenesis under certain circumstances. Direct injection of MSCs into an in vitro Matrigel angiogenesis assay led to the induction of apoptosis in endothelial cells. This assay showed that endothelial apoptosis was accompanied by increase in reactive oxygen species, which ultimately led to capillary degeneration. Further, direct in vivo injection of MSCs into mouse melanomas exhibited tumor devascularization via a reduction in endothelial markers PECAM1 and VE-cadherin [74].

Additional research has demonstrated the anti-angiogenic effects of MSCs in gliomas. Bone marrow-derived MSCs suppress the growth of both patient-derived primary glioma cells in vitro and human glioma cell lines in vivo. Co-injection of human-derived MSCs and glioma cell lines resulted in a significant reduction of microvessel density, as demonstrated with CD31 staining. Further proteomic analysis of these samples showed downregulation of the pro-angiogenic factors PDFG-BB, IGF-1, FGF-2, and IL-1β. In vivo glioma-MSC co-cultures also demonstrated a decrease in PDGF-BB and IL-1β expression and a reduction in tumor volume compared to glioma-only tumors [75].

Given the data presented in mouse melanomas and human gliomas, MSCs may play a role in both the enhancement and inhibition of angiogenesis.

3.3.3 Regulation of Cellular Signaling

Within the tumor microenvironment, various cellular signals regulate tumor cell survival, proliferation, migration, and metabolism. Increasing evidence shows that MSCs influence the cellular signaling of tumor cells. In addition to pro-tumorigenic regulation, MSCs regulate signaling pathways that inhibit tumor progression. The phosphoinositide 3-kinase/AKT and WNT/β--catenin signaling pathways are associated with the development of carcinomas of the breast, liver, colon, skin, stomach, and ovary. Studies report that MSCs inhibit tumor proliferation through inhibition of the PI3K/AKT pathway and suppression of the WNT/β-catenin pathway. MSCs specifically induced expression of DKK1 in human carcinoma cell lines (hepatocellular, H7402 and HepG2; breast, MCF-7; hematopoietic, K562 and HL60) via WNT signaling, which inhibited cell proliferation [76–78].

3.3.4 Induction of Apoptosis

MSCs have also been reported to induce tumor cell apoptosis and cell cycle arrest [79]. MSCs had an inhibitory effect on mouse hepatoma, lymphoma, and insulinoma cells through induction of p21 and the caspase 3 pathway [80]. Moreover, MSCs cultured at a high density expressed type I IFN, leading to the cell death of breast cancer cells, MCF-7, and MDR-MB-231 cells. Furthermore, MSCs primed with IFN-γ can induce tumor cell-specific apoptosis [81, 82] (Fig. 3.3).

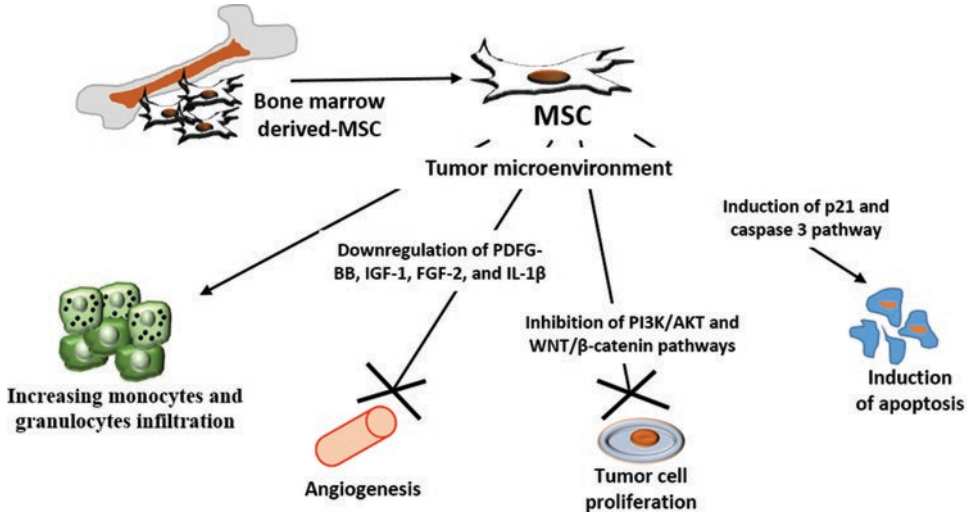

Fig. 3.3 The role of MSCs in suppressing tumor progression through increasing monocyte and granulocyte infiltration, inhibiting angiogenesis and tumor cell proliferation, and inducing tumor cell apoptosis

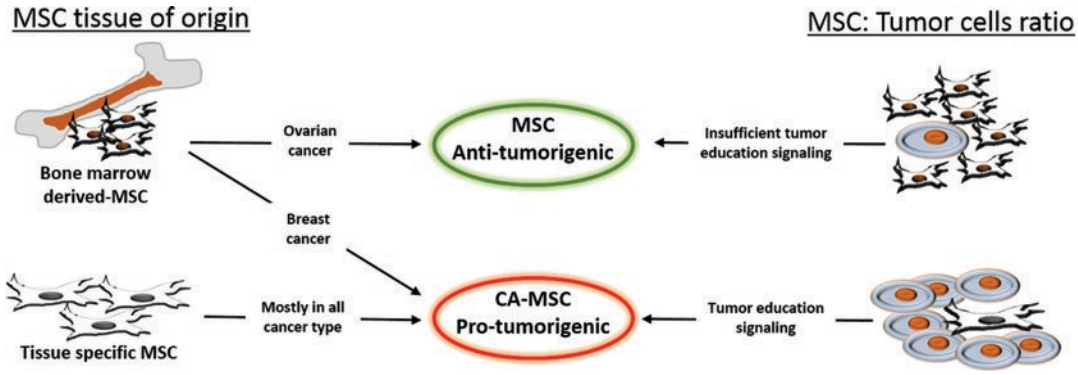

Fig. 3.4 MSCs source and number affect the role of MSCs within tumor microenvironment into pro-tumorigenic versus anti-tumorigenic

3.4 Conclusions

While examples of MSCs functioning in an anti-tumorigenic manner exist, the majority of evidence points to MSCs acting in a cancer supporting role within the confines of the TME. These antitumorigenic findings cannot be merely discarded however, but rather contextualized. Tumor-suppressing effects are observed in higher ratios of MSCs to tumor cell (~2:1 and greater) which are significantly greater than the TME MSC population [74, 83, 84]. These findings support the development and use of ex vivo MSCs in a therapeutic role but lack the physiological relevancy representative of the natural TME (Fig. 3.4).

MSC/CA-MSC function also appears to develop in a tissue- and disease-dependent manner. Bone marrow-derived MSCs (BM-MSCs) developed a cancer supporting phenotype in a breast cancer TME model but not an ovarian cancer TME model. However, omental-derived MSCs were able to promote growth in the ovarian cancer TME model, while BM-MSCs inhibited tumor growth in the ovarian cancer TME model [85]. As breast cancer typically metastasizes to bone while ovarian cancer rarely does

and prefers to metastasize to omentum, these findings suggest the importance in MSC source in the development of tumor supporting/suppressing phenotypes and may explain some of the divergent findings regarding MSC function. Further, most of the reports demonstrating antitumorigenic roles for MSCs are from experiments using MSCs without prior exposure to cancer cells or without direct association with cancer cells. This speaks to an important difference in the function of cancer-naïve MSCs vs cancer-educated MSCs.

Despite the divergence in evidence describing the role of MSCs in tumor promotion or suppression, it is apparent that MSCs play a dynamic role within the TME. Further work is required to unravel the complex crosstalk between MSCs and tumor, immune, and other stromal cells. Given the heterogeneity of MSCs, additional work is required to identify and adequately describe various subpopulations that may have differing functions dependent on cancer type. This will be essential to understanding how MSCs contribute to cancer development and progression and may lead to the identification of new therapeutic targets or biomarkers as well as the use of MSCs as therapeutic agents.

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Hepatic Stellate Cells in Liver Tumor

4

Hidenori Shiraha, Masaya Iwamuro, and Hiroyuki Okada

Abstract

Hepatocellular carcinoma and intrahepatic cholangiocarcinoma are the most common types of primary liver cancers. Moreover, the liver is the second most frequently involved organ in cancer metastasis after lymph nodes. The tumor microenvironment is crucial for the development of both primary and secondary liver cancers. The hepatic microenvironment consists of multiple cell types, including liver sinusoidal endothelial cells, Kupffer cells, natural killer cells, liver-associated lymphocytes, and hepatic stellate cells (HSCs). The microenvironment of a normal liver changes to a tumor microenvironment when tumor cells exist or tumor cells migrate to and multiply in the liver. Interactions between tumor cells and non-transformed cells generate a tumor microenvironment that contributes significantly to tumor progression. HSCs play a central role in the tumor microenvironment crosstalk. As this crosstalk is crucial for liver carcinogenesis and liver-tumor development, elucidating the mechanism underlying the interaction of HSCs with the tumor microenvironment could provide potential therapeutic targets for liver cancer.

Keywords

Extracellular matrix · Matrix metalloproteinase · Myofibroblast · Cancer-associated fibroblast · Tumor-infiltrating leukocyte · Platelet-derived growth factor · Transforming growth factor-β · Epithelial-mesenchymal transition · Vascular endothelial growth factor · Tumor-associated macrophages · Stromal cell-derived factor-1 · Tumor stroma · Jagged-1 · Angiogenesis · Fibroblast activation protein

Abbreviations

CAFs	Cancer-associated fibroblasts
CCL2	Chemokine (C-C motif) ligand 2
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FAP	Fibroblast activation protein
FGF	Fibroblast growth factor
GI tract	Gastrointestinal tract
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HSCs	Hepatic stellate cells
ICC	Intrahepatic cholangiocarcinoma
IGF-I	Insulin-like growth factor I
IL	Interleukin
LSECs	Liver sinusoidal endothelial cells
MCP1	Monocyte chemoattractant protein 1
MDSCs	Myeloid-derived suppressor cells

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_4

MFB	Myofibroblast
MMPs	Matrix metalloproteinases
NK cell	Natural killer cell
OPN	Osteopontin
PDGF	Platelet-derived growth factor
SDF-1	Stromal cell-derived factor-1
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor-β
TILs	Tumor-infiltrating leukocytes
TIMPs	Tissue inhibitors of matrix metalloproteinases
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
α-SMA	α-smooth muscle actin

4.1 Introduction

4.1.1 Hepatic Stellate Cells (HSCs)

The hepatic microenvironment consists of multiple cell types, including liver sinusoidal endothelial cells (LSECs), Kupffer cells, natural killer (NK) cells, liver-associated lymphocytes, and hepatic stellate cells (Fig. 4.1). HSCs, also known as perisinusoidal cells or Ito cells, are liver-specific mesenchymal cells located in perisinusoidal and portal areas. They constitute approximately 15% of the total liver-cell number. The characteristic feature of HSCs in the normal liver is the storage of vitamin A in lipid droplets. Lipid droplets are important for remodeling the extracellular matrix (ECM) by producing both the ECM and matrix metalloproteinases (MMPs). HSCs also produce growth factors and cytokines. There are two main phenotypes of HSCs: "quiescent" and "activated," and their development depends on the physiological condition of the liver. Liver injury induces the activation of HSCs and is characterized by enhanced proliferation and formation of myofibroblast (MFB)-like cells. Activated HSCs are the major source of ECM components, including collagen and proteoglycans. Furthermore, HSCs are important for creating an environment for the development of hepatic progenitor cells and hepatocytes.

4.1.2 Liver Tumors

Liver cancers can be primary or secondary. Primary liver cancers include hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined hepatocellular carcinoma and cholangiocarcinoma. HCC and ICC are the two major types of primary liver tumors. Hepatocellular carcinoma is the most common primary liver cancer and the fourth leading cause of cancer-related deaths worldwide [1, 2]. It accounts for 90% of all cases of primary liver cancer [3]. Chronic infection with hepatitis B or hepatitis C virus is the major cause of HCC; other causes include heavy alcohol use, autoimmune liver diseases, and nonalcoholic steatohepatitis. Continuous inflammation occasionally damages the DNA in the hepatocytes of a regenerating liver, thereby increasing the chances of gene alterations leading to carcinogenesis. In an HCC tumor, not only tumor cells but also several stromal cells, including HSCs, LSECs, cancer-associated fibroblasts (CAFs), Kupffer cells, and tumor-infiltrating leukocytes (TILs), are present (Fig. 4.2).

Intrahepatic cholangiocarcinoma is the second most common type of primary liver malignancy, accounting for 5% of primary liver cancers. It is an aggressive type of cancer and has a poor prognosis, as therapeutic strategies against ICC are limited. Chemotherapy and radiotherapy are not highly effective as ICC exhibits a fibrous stroma that is resistant to these treatments. Furthermore, the neoplastic transformation, progression, metastasis, and invasion of ICC are caused by the tumor microenvironment, which contains HSCs, LSECs, CAFs, Kupffer cells, and TILs (Fig. 4.3).

Most cases of secondary liver cancer are liver metastases. Liver metastases are tumors that have spread to the liver from other areas of the body. They are more common than primary liver cancers [1, 4]. The liver is the most common site of metastatic spread after the lymph nodes [5]. Liver metastasis is common in many types of cancer, including breast, cervical, and lung cancer [6]. Gastrointestinal (GI) tract and pancreatic malig-

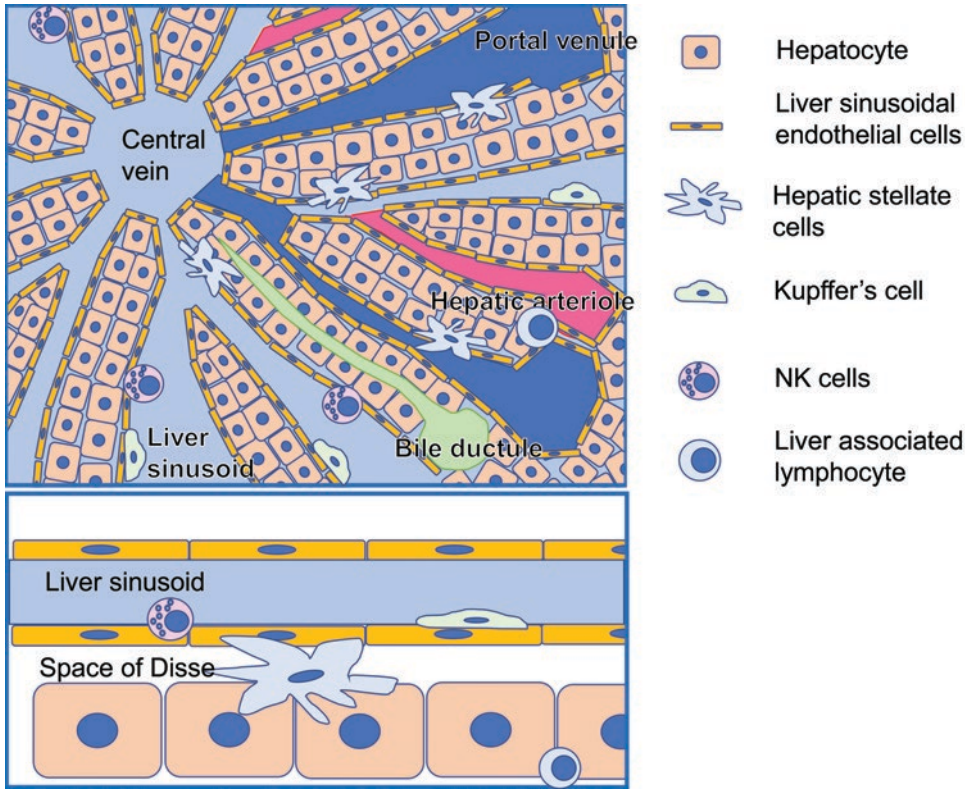

Fig. 4.1 The structure of a liver lobule. Hepatic stellate cells (HSCs) are located in the space of Disse between hepatocyte and liver sinusoidal endothelial cells. Kupffer cells and liver associated lymphocytes, including NK cells, are mainly located in hepatic sinusoid

nancies can also easily spread to the liver, presumably because of hepatic portal venous drainage from the GI tract and pancreas [6].

The hepatic microenvironment is a complex system, consisting of the ECM and soluble cytokines, apart from the hepatic cells. Under physiological conditions, the hepatic microenvironment protects hepatic cells from malignant transformation by regulating cell proliferation and providing cell polarity. Upon liver metastasis, the hepatic microenvironment changes into a tumor microenvironment. Interactions between tumor cells and non-transformed cells generate a tumor microenvironment that contributes significantly to tumor progression. In this section, the role of the tumor microenvironment during tumor progression and metastasis to the liver will be discussed.

4.2 Hepatic Stellate Cells in Hepatocellular Carcinoma

4.2.1 Role of HSCs in HCC

The progression of HCC is regulated by the hepatic microenvironment (Fig. 4.2) [7, 8]. HSCs are present within HCC tissues, as demonstrated histopathologically [9]. HSCs and activated MFBs infiltrate the stroma of HCC and localize around tumor sinusoids [10–12]. HSCs are involved in the production of cytokines, chemokines, growth factors, ECM, and MMPs. The hepatic microenvironment primarily consists of ECM proteins and proteoglycans produced by stromal cells. Activation of stromal cells results in ECM remodeling. Hepatocellular carcinoma primarily develops from chronic hepatic diseases

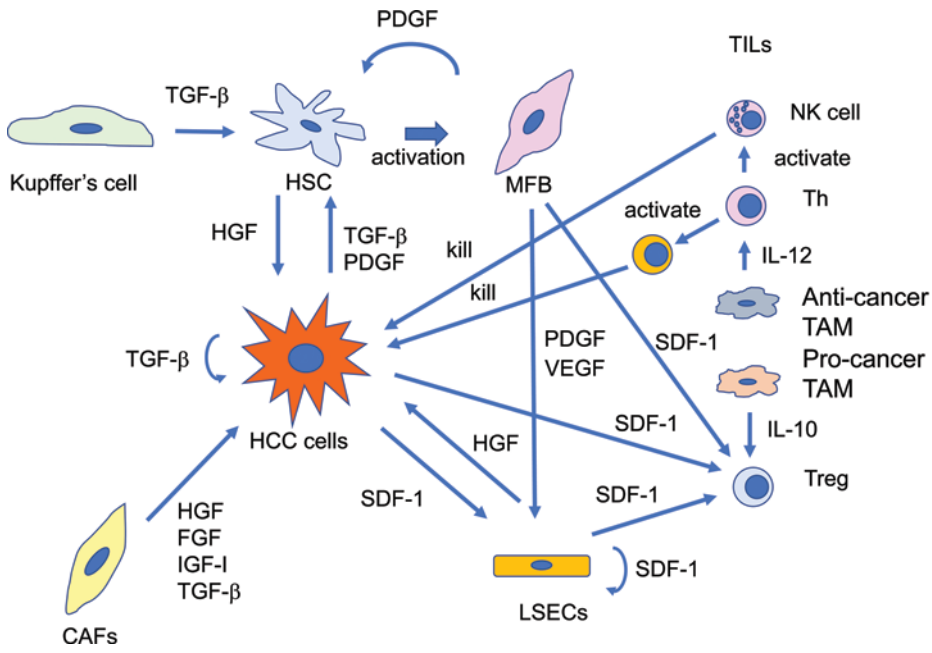

Fig. 4.2 HCC tumor microenvironment. The HCC tumor microenvironment consists of stromal cells, including Kupffer cells, HSCs, CAFs, LSECs, TAMs, and lymphocytes. The HCC-HSC crosstalk plays a pivotal role in the development and progression of HCC. Each of the cell-cell interactions is symbolized by arrows

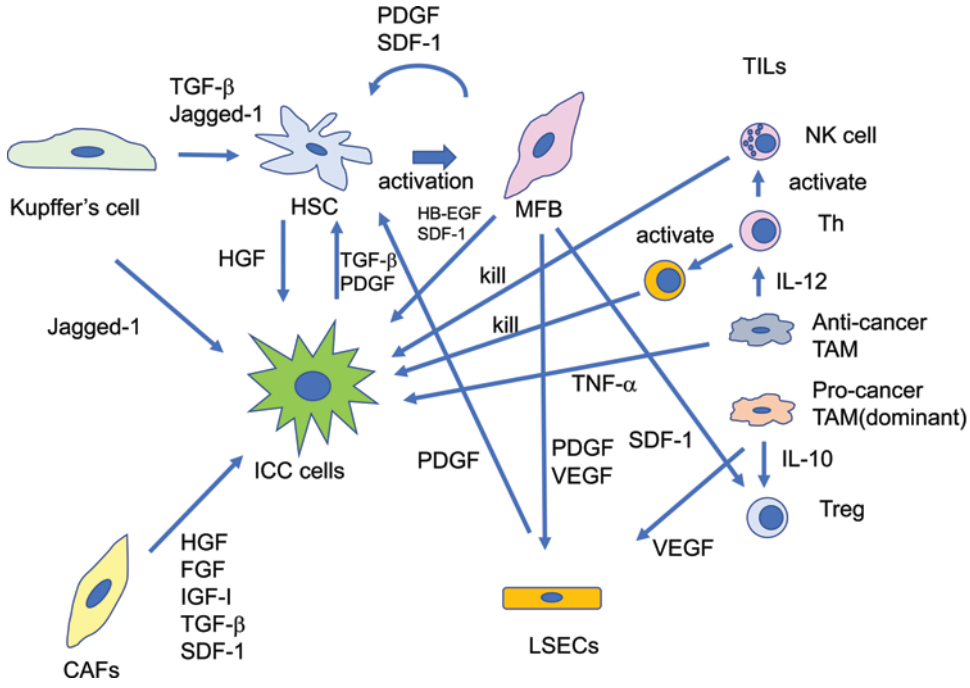

Fig. 4.3 ICC tumor microenvironment. The interactive network of ICC and the tumor microenvironment cells are shown. HSCs play a central role in the development and progression of ICC, especially in the cytokine crosstalk between ICC and stromal cells

involving inflammation, which causes ECM deposition [13]. Furthermore, HSCs produce proteolysis-resistant collagens, reducing ECM degradation [14, 15]. Additionally, the increase in the levels of tissue inhibitors of MMPs (TIMPs) enhances ECM accumulation. Abnormal ECM accumulation stimulates HCC progression.

Hepatic stellate cells have a heterogeneous function and influence HCC progression. Some functions of HSCs are tumor promoting. Media conditioned with activated HSCs has been reported to induce the proliferation and migration of HCC cells [11]. HSCs become activated MFBs in response to platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β [16, 17]. The PDGF-C transgenic mouse demonstrated activation and proliferation of HSCs and development of HCC [18].

A co-transplant model of HCC cells and HSCs has been used to investigate the interactions between these cells [19, 20]. These studies demonstrated that TGF-β signaling interference reduced the development of HCC in vivo. Furthermore, HCC cells frequently produced TGF-β in an autocrine manner [21–23]. Another study demonstrated that HSCs promoted epithelial-mesenchymal transition (EMT) in HCC cells via TGF-β [24]. Thus, TGF-β has been implicated as the key signaling molecule involved in the interaction between HCC cells and HSCs.

The downregulation of the expression levels of TGF-β receptors in HCC compared with those in the adjacent normal tissues established the importance of TGF-β signaling in the initiation of HCC [25]. Hepatic tumor-initiating cells may be derived from hepatic progenitor cells exposed to chronic TGF-β stimulation in cirrhotic liver [26]. As hypoxic hepatocytes secrete enzymes that activate latent TGF-β, hypoxia induces EMT in hepatocytes in a TGF-β-dependent manner [26]. TGF-β induces the development of a cancer microenvironment through the generation of CAFs, which produce growth factors and cytokines [27–29]. Additionally, TGF-β is responsible for the activation of HSCs to activate MFBs.

Another role of HSCs in HCC development is the promotion of angiogenesis. When HSCs are co-cultured with HCC cells, the expression of pro-angiogenic genes, such as the vascular endothelial growth factor (VEGF)-A and MMP-2, in HSCs as well as HSC proliferation and migration are enhanced by HCC cells [30].

The complex crosstalk between HSCs and other liver cells, including HCC, is important for the development of HCC.

4.2.2 Kupffer Cells

Kupffer cells are present in the sinusoidal cavity, and they adhere to LSECs. Kupffer cells are macrophages derived from the bone marrow that migrate to the liver and exhibit phagocytic and antigen-presenting functions. They produce cytokines, including TGF-β, which induce HSCs from a quiescent state to an activated state [31]. Moreover, Kupffer cells contribute to the pool of tumor-associated macrophages (TAMs) and bone-marrow-derived macrophages [32].

4.2.3 LSECs

During the development of HCC, a switch to arterial blood supply occurs. The predominant blood supply at the early stage of HCC is arterial. Hypoxia-inducible factors, including VEGF, promote vascularization in HCC [33–36]. During the blood supply transition, hepatic sinusoids undergo capillarization, thereby causing the loss of sinusoidal fenestrae and the development of a basement membrane [37, 38]. Liver sinusoidal endothelial cells, present in HCC, sequentially diminish during the development of HCC, causing the loss of LSEC markers, including stabilin-1, stabilin-2, LYVE-1, and CD32b [39].

The chemokine stromal cell-derived factor-1 (SDF-1), also known as CXCL12, is constitutively expressed in normal liver. SDF-1 is produced by biliary epithelial cells, HSCs, and LSECs in the liver [40]. It is involved in tumor progression as well as liver inflammation and liver regeneration. SDF-1 activates two chemokine receptors, CXCR4 and CXCR7 [41]. The expression of CXCR4 is related to the recruitment

of regulatory T cells (Treg) in tumors [42–45]. In a clinical study, high CXCR4 expression was associated with tumor progression and metastasis [46–49]. Although CXCR7 was activated by SDF-1 in HCC cell lines, the expression of CXCR7 was not significantly related to the prognosis of patients with HCC [47]. Additionally, SDF-1 is involved in angiogenesis, as HIF-1 and VEGF upregulate CXCR4 [50].

LSECs are a source of hepatocyte growth factor (HGF), which induces hepatocyte regeneration. An HSC-derived PDGF activates VEGFR1 and VEGFR2 of LSECs, resulting in the release of HGF [51–54]. HGF, which is a member of the epidermal growth factor family, is produced by CAFs, HSCs, and MFBs and stimulates cell proliferation, migration, and angiogenesis [55–60]. The activation of c-Met, an HGF receptor, causes the downstream activation of the mitogen-activated protein kinase phosphoinositide-3 kinase and rac-cdc42 pathways. The activation of these signaling pathways contributes to tumor cell proliferation, migration, and survival. HGF enhances angiogenesis directly and indirectly by inducing VEGF [61]. Moreover, activated HSCs promote angiogenesis in HCC [62–64]. The interactions between activated HSCs and LSECs contribute to the establishment of the tumor microenvironment.

4.2.4 CAFs

Cancerous tumors consist of heterogeneous cancer and stromal cells. The stromal cell-associated cancer microenvironment is critical for cancer growth and progression [65, 66]. HSCs secrete various growth factors and directly regulate hepatocytes. The presence of HSCs in the stroma of HCC has been reported by an immunohistochemical analysis [11]. Activated peritumoral HSCs have been associated with tumor recurrence and mortality [67]. Cancer-associated fibroblasts constitute the major population in the HCC stromal and secrete a variety of cytokines, including TGF-β, HGF, fibroblast growth factor (FGF), and insulin-like growth factor I (IGF-I) [68–71]. These cytokines induce cancer growth and progression. Most cases of HCC develop from liver cirrhosis, in which fibroblasts are activated because of chronic liver inflammation. The cytokine-array analysis of isolated CAFs revealed that the HGF was the most prominent CAF-derived cytokine activating HCC cells [69].

4.2.5 TILs

Several studies demonstrated that TILs could be a prognostic biomarker in HCC [72–75]. Tumor-infiltrating lymphocytes consist of T cells, B cells, NK cells, and macrophages. Tumor-associated macrophages are the major component of TILs, originating from circulating monocytic precursors.

The infiltration of lymphocytes is related to the prognosis of HCC patients. Patients positive for CD3, a surface antigen of T lymphocytes and TILs, demonstrated better prognosis [76]. T lymphocytes are important for the antitumor immune response. High densities of intratumoral cells positive for CD8, a surface antigen for cytotoxic T lymphocyte, are associated with survival in HCC [7, 77, 78]. Activated HSCs have been reported to suppress $CD8^+$ T cell proliferation and IFN-γ production [79]. Additionally, they inhibited T-cell response by inducing T-cell apoptosis [80]. The accumulation of cells with immune-suppressive activities, like myeloid-derived suppressor cells (MDSCs) and Tregs, is a key mechanism for tumor immune evasion [81, 82]. Furthermore, activated HSCs were shown to enhance immunosuppressive cell populations, including those of Treg and MDSCs [83].

The number of infiltrating NK cells correlated with HCC cancer cell apoptosis and patient survival [78, 84]. NK cells were shown to kill activated HSCs directly and induce HSC apoptosis by the production of IFN-γ [85, 86].

Tumor-associated macrophages have a dual role in cancer progression and can be classified as anti-cancer TAMs and pro-cancer TAMs [87]. Anti-cancer TAMs can be activated by interferon-γ and increase the expression of interleukin (IL)-12, which activates T helper lymphocytes [88, 89]. Meanwhile, IL-4, IL-10, and

IL-13 can convert macrophages into pro-cancer TAMs. Pro-cancer TAMs exhibit poor antigen-presenting capability and produce cytokines and chemokines, including IL-10 and TGF-β. The TGF-β produced by TAMs stimulates HSCs to transdifferentiate into MFBs. Subsequently, the TGF-β produced by TAM and MFBs induces the progression of hepatocytes to neoplastic hepatocyte.

4.3 Hepatic Stellate Cells in Intrahepatic Cholangiocellular Carcinoma

Studies regarding the tumor microenvironment in ICC are scarce compared with those regarding the HCC tumor microenvironment. The desmoplastic stroma surrounding ICC cells is important for the development of ICC. The tumor microenvironment of ICC is composed of stromal cells, including HSCs, LSECs, CAFs, Kupffer cells, and TILs (Fig. 4.3). These cells contribute to tumor progression by secreting various soluble factors. These factors directly enhance ICC cell proliferation and migration as well as induce the aberrant activation of other stromal cells [90, 91].

ICC cell migration and survival were modulated by SDF-1 released by HSCs [92]. Additionally, SDF-1 enhances EMT through the interaction between activated HSCs and the SDF-1/CXCR4 axis in ICC [93]. Furthermore, SDF-1 activates HSCs in an autocrine manner.

Sulpice et al. demonstrated a significant genomic change in ICC stromal cells [94]. Upregulated genes in the stroma of ICC were related to the cell cycle, ECM, and TGF-β. Furthermore, it was demonstrated that the stromal expression of osteopontin (OPN) was closely related to ICC prognosis. Since OPN contributes to TGF-β-mediated HSC activation [95], activated HSCs could be involved in ICC progression through OPN.

High expression of the ECM was associated with poor prognosis in ICC [96–98]. The major sources of ECM in ICC could be CAFs and HSCs. Surgically resected tumors from patients with ICC show a high expression of α-smooth muscle actin (SMA), a marker of HSC, and poor survival compared to low-α-SMA-expression tumors [96, 97]. The co-culture of an ICC cell line with an HSC line increased the cell proliferation and invasion of ICC cells [97]. Another study demonstrated that an HSC line induced the proliferation, migration, and invasion of ICC cells via hedgehog signaling [99]. These data suggest that HSCs are involved in the promotion of ICC.

4.3.1 Kupffer Cells

Kupffer cells produce TGF-β and activate HSCs in the tumor microenvironment of ICC. In a study of ICC using an animal model, Kupffer cell transiently congregated around the central veins in the liver and expressed the Notch ligand Jagged-1, activating Notch in the pericentral hepatocytes [100]. Notch signal activation is required for ICC progression through the deactivation of p53 [101]. Additionally, Jagged-1 activates HSCs, enhances α-SMA and collagen production, and contributes to the formation of tumor stroma [102].

4.3.2 LSECs

Liver sinusoidal endothelial cells are important for the activation of TGF-β through plasmin. Subsequently, activated TGF-β mediates the activation of HSCs. LSECs secrete PDGF, and HSCs are activated in a paracrine and autocrine manner. Activated HSCs produce PDGF ligands and angiopoietins and enhance angiogenesis in ICC [103].

4.3.3 CAFs

The stroma of ICC tumors contains a number of CAFs that produce abundant ECM. Although CAFs form the majority of stromal cells, the origin of CAFs is still unclear. The CAF population is heterogeneous, and CAFs potentially originate from HSCs, portal fibroblasts, bone-marrow-derived fibroblasts, and transformed ICC cells [90]. The

TGF-β released from ICC cells has been reported to induce HB-EGF expression in MFBs [104]. Conditioned medium from human-ICC-derived CAFs promoted the proliferation of ICC cell lines in both a paracrine and juxtacrine manner [96].

CAFs are a major source of SDF-1 in ICC. The high stromal expression of SDF-1 predicted a poor prognosis for patients with ICC [93]. SDF-1, along with TAM-derived TNF-α, stimulates CXCR4 expression in ICC cells, resulting in a hyper-response to SDF-1 [105].

4.3.4 TILs

Similar to the HCC tumor microenvironment, TILs consist of T cells, B cells, NK cells, and macrophages. NK cells are critical in the innate immune defense against ICC. Adoptive NK cells have demonstrated cytolytic activity against ICC cells in a nude mouse model [106].

TAMs, CAFs, and cancer cells produce monocyte chemoattractant protein 1 (MCP1), also known as chemokine (C-C motif) ligand 2 (CCL2), which causes T cells to express CD4/CD25 and, subsequently, become Treg [107].

Tumor-associated macrophages are primarily derived from the bone marrow, rather than the resident macrophages or Kupffer cells. They are divided into pro-cancer and anti-cancer TAMs. TAMs activated with TNF-α have an anti-cancer activity. Most TAMs in ICC are pro-cancer TAMs, which produce angiogenic factors, such as VEGF and IL-10 [90]. High macrophage density predicted a poor prognosis for patients with ICC [108–110]. Additionally, activated HSCs promoted the differentiation of liver macrophages with a pro-cancer phenotype [111].

4.4 Tumor Microenvironment in Metastatic Liver Tumor

The liver is the second most frequently affected organ in cancer metastasis after the lymph nodes. The hepatic microenvironment determines tumor cell dormancy and metastatic outgrowth (Fig. 4.4). Circulating metastatic cells can enter the liver through both the portal vein and hepatic artery. Liver-infiltrating cancer cells are entrapped in the sinusoids and can lead to cell death or survival following extravascular migration. NK cells are critical for killing infiltrating cancer cells [74]. Additionally, Kupffer cells kill cancer cells by phagocytosis [112–114].

Stromal cells are recruited in avascular micrometastasis. Similar to the development of HCC, stromal cells are important for the development of metastatic liver tumors. Pre-metastatic niches are formed by the recruited bone-marrow-derived stromal cells [115]. These niches consist of $CD11b^{+}/VEGFR1^{+}$ cells [116]. There have been reports showing that bone-marrow-derived cells promoted the development of liver metastasis of colorectal cancer [117]. Bone-marrow-derived cells play an important role in establishing metastasis in pre-metastatic niches. HSCs are activated and transdifferentiated into MFBs by paracrine factors released by both cancer cells and LSECs [118, 119]. HSCs are important for the pre-metastatic niche formation for liver metastasis in pancreatic cancer. The expression level of fibronectin by HSCs increases markedly [2], while MFBs primarily release PDGFs, HGF, and TGF-β. These factors initiate angiogenesis.

Endothelial cells are recruited in response to angiogenic factors released from the stromal cells, leading to tumor vascularization. The recruited endothelial cells contribute to blood vessel formation. Micrometastasis develops upon the co-localization of MFBs and endothelial cells. Additionally, hypoxia stimulates MFBs, thus causing them to produce angiogenic factors [120]. HSCs also contribute to the establishment of liver metastasis via inflammatory-response -related mechanisms [118, 120, 121].

Tumor cells grow and metastasize. Once clinical metastasis is established, the tumors start growing aggressively. Angiogenic alteration occurs from the portal vein to the hepatic artery [122]. As tumor cells proliferate, ECM degradation is required for tumor expansion. The degradation of the surrounding ECM barriers allows tumor cells to grow expansively. MMPs are critical for ECM degradation. Additionally, MMPs release active growth factors and promote angiogenesis [123].

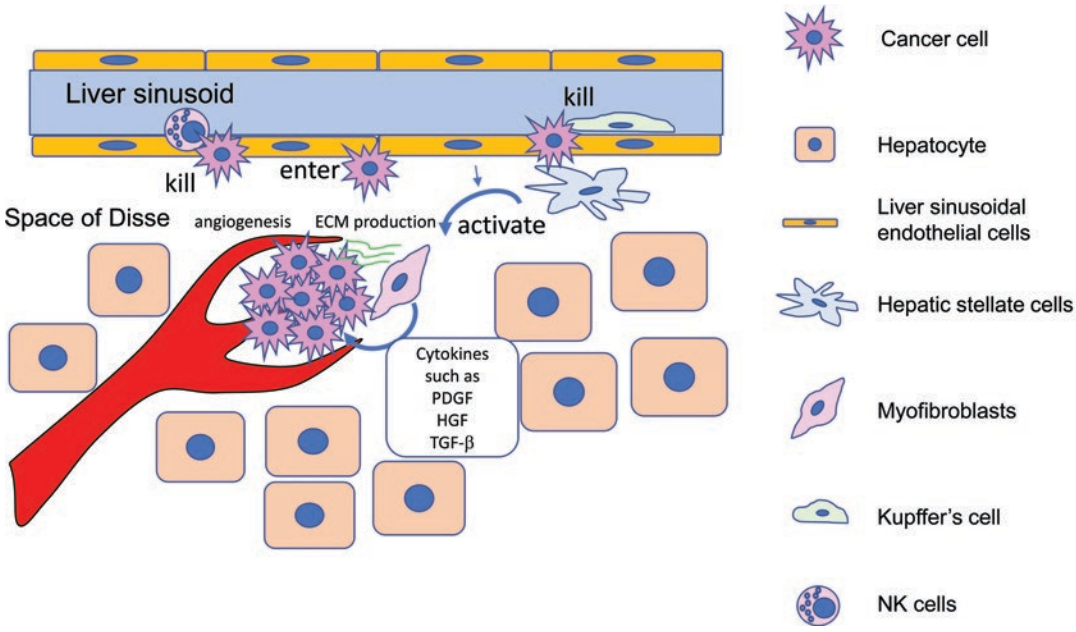

Fig. 4.4 Tumor microenvironment in liver metastasis. Shown are the major cell types of the tumor microenvironment in liver metastasis. Metastatic cancer cells enter from a sinusoid. Most of the cancer cells are trapped and killed by Kupffer cells and NK cells. The escaped cancer cell forms micrometastasis and induces a pro-metastatic microenvironment. HSCs promote metastatic growth by ECM production and cytokine secretion, which enhances tumor cell growth and angiogenesis

4.5 Commentary on Likely Future Trends and Directions

The therapeutic modalities for liver tumors primarily target cancer cells. Chemotherapy is the most common form of treatment against cancer cells. The development of chemoresistance and the destruction of a patient's immune system are the major problems involved in cancer chemotherapy. HCC development is caused not only by the genetic mutation of hepatocytes but also by the liver microenvironment. Interactions between the tumor cells and liver microenvironment cause both proliferation and suppression of tumor cells. The microenvironment of the liver tumor has not been fully characterized. The mechanism underlying the crosstalk between tumor cells and stromal cells in the tumor microenvironment may be characterized to develop novel therapies targeting the tumor microenvironment associated with HCC and other liver tumors.

A possible target for the treatment of liver tumor could be activated HSCs, as they are critical for the tumor microenvironment. Sibrotuzumab, a humanized monoclonal antibody against the fibroblast activation protein (FAP), could be used for targeting activated HSCs [124]. FAP is a membrane-bound gelatinase, and its expression has been detected in fibrotic liver but not in normal human liver. Additionally, it is co-localized with α-SMA in vivo and with isolated HSCs in vitro, suggesting its expression in activated HSCs [125]. Although sibrotuzumab has not been used in clinical trials for HCC or ICC, it could be a potential therapeutic agent targeting their tumor microenvironment. ValboroPro could be another potential therapeutic agent for inhibiting FAP [126].

Another potential target could be the hedgehog signaling pathway. This pathway is a key regulator of animal development and is present in all bilaterians [127]. Mammals have three

hedgehog homologs: Desert, Indian, and Sonic. The hedgehog signaling pathway is activated when the hedgehog ligand binds to Patched. Hedgehog signaling regulates the fate of HSCs by regulating metabolism [127]. Inhibiting hedgehog signaling could inhibit the activation of HSCs. Furthermore, hedgehog signaling is involved in the progression of EMT in HCC and ICC [128, 129]. The inhibition of hedgehog signaling by cyclopamine and capsaicin impaired EMT in ICC [129, 130]. These findings further demonstrate the potential of novel therapeutic strategies targeting the tumor microenvironment.

As the tumor microenvironment is important for the development of liver tumors, therapeutic strategies targeting the components of their tumor microenvironment have been developed [131]. Further studies are necessary to develop therapeutic strategies targeting the tumor microenvironment. A combination therapy employing cytotoxic agents and targeting of the tumor microenvironment could be a viable therapeutic strategy for liver cancer. Furthermore, cytotoxic agents are currently being replaced with monoclonal antibodies and small-molecule kinase inhibitors.

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5 Pancreatic Stellate Cells: The Key Orchestrator of The Pancreatic Tumor Microenvironment

Divya Thomas and Prakash Radhakrishnan

Abstract

Pancreatic cancer is one of the most challenging adenocarcinomas due to its hostile molecular behavior and complex tumor microenvironment. It has been recently postulated that pancreatic stellate cells (PSCs), the resident lipid-storing cells of the pancreas, are important components of the tumor microenvironment as they can transdifferentiate into highly proliferative myofibroblasts in the context of tissue injury. Targeting tumor-stromal crosstalk in the tumor microenvironment has emerged as a promising therapeutic strategy against pancreatic cancer progression and metastasis. This chapter brings a broad view on the biological and pathological role of PSCs in the pancreas, activated stellate cells in the onset of tissue fibrosis, and tumor progression with particular emphasis on the bidirectional interactions between tumor cells and PSCs. Further, potential therapeutic regimens targeting activated PSCs in the pre-clinical and clinical trials are discussed.

Keywords

Pancreatic stellate cells · Pancreatic cancer · Stroma · Desmoplasia · Fibrosis · Cancer-associated fibroblast · TGFβ · Wnt signaling · Drug resistance · Tumor microenvironment

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5.1 Introduction

With the limited advancement in therapy, pancreatic cancer (PC) is predicted to become the second leading cause of cancer-related death within the next decade in Western countries [1]. Pancreatic ductal adenocarcinoma (PDAC) is the most common (~95%) type of pancreatic cancer. Emerging research approaches in the genetic and epigenetic alterations, tumor-stromal crosstalk, and identification of early detection biomarkers

A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_5

has not yet ensured a dramatic change in the overall survival of PC patients [2]. Moreover, 0.3% increment in the death rate of PC patients has been reported during 2011 through 2015 [3] with the lifetime chances of developing PC that is approximately 1 in 64 individuals. The inefficiency of the current experimental models in recreating tumor microenvironment and desmoplasia which is comprising about 80% of the tumor mass results in an inconsistency between experimental results and clinical outcomes [4]. After decades of research in the epithelial and stromal components of the tumor, it is clear that dense fibrotic stroma is not just a bystander but an active player during PDAC progression. The cross-talk between tumor and stromal compartments are still complex and the actual function of tumor surrounding dense stroma remains largely unknown until the use of pancreatic stellate cells in research [5, 6]. Distinct types of cells in the tumor microenvironment such as pancreatic stellate cells (PSCs), cancer-associated fibroblasts (CAF), endothelial cells, immune cells, nerve cells, and extracellular matrix (ECM) are involved in the induction of desmoplastic reactions in the pancreas. This microenvironment undergoes dynamic alterations that drive PDAC tumor progression in cooperation with several other oncogenic signaling cascades [7]. Stromal alterations are primarily driven by the activation of tissue-resident PSCs, and therefore it is considered as the sprouted seed for PDAC progression [8, 9]. In this chapter, we provide a detailed perspective on the biological importance of PSCs in stromal activation and PDAC progression. A better understanding of the dynamic interplay between tumor and stroma may represent an innovative field of research where new drugs targeting stromal alterations could be developed.

5.2 Pancreatic Stellate Cells: An Overview

The existence of PSCs exhibiting abundant vitamin-A-containing lipid droplets that reside in the periacinar and interstitium of the pancreatic tissue was first described in 1982 [10]. However, PSCs were first isolated and cultured nearly two decades later only by two independent research group [5, 6], which opened up an avenue in the field of pancreatic fibrogenesis. The origin of PSCs remains unresolved; however endodermal, mesenchymal, neuroectodermal, and bone marrow-derived cell origins of PSCs have been described [11, 12]. Another study suggests the possibility for the risen up of PSCs from C-C chemokine receptor 2 (CCR2) (+) monocytes that migrate into the pancreas [13]. In the healthy pancreas, PSCs appear in their quiescent phenotype which is stagnant and almost redundant or little is known about its physiological functions. The quiescent or inactivated PSCs contain retinoid and therefore these are vital for maintaining tissue homeostasis. Metabolites of retinol are known to mediate physiological functions such as protein synthesis, cell proliferation, and differentiation [14]. Interestingly, the maintenance of quiescent phenotype of PSCs has been shown to be dependent on the level of vitamin A as it inhibits the expression of α-smooth muscle actin (α-SMA), collagen, fibronectin, and laminin [15]. The structure of quiescent PSCs resembles rough endoplasmic reticulum and is plenty of collagen fibrils and vitamin-A-containing lipid droplets surrounding the central nucleus. Quiescent PSCs have the ability to produce ECM proteins such as desmin, vimentin, and matrix-degrading enzymes such as matrix metalloproteinases (MMPs). Also, it has the ability to produce tissue inhibitors of MMPs (TIMPs); hence, PSCs are thought to play an important role in maintaining the balance between matrix formation and degradation and therefore maintaining the normal tissue architecture [16]. However, any environmental/external stimuli result in the activation of PSCs which is transformed into myofibroblast-like phenotype. This phenotypical transition is correlated with functional and morphological changes including loss of vitamin-A-containing lipid droplets; increased expression of α-SMA; increased production of collagen, laminin, nestin, and fibronectin; decreased production of desmin and vimentin; increased production of ECM; enlarged nucleus; loss of balance

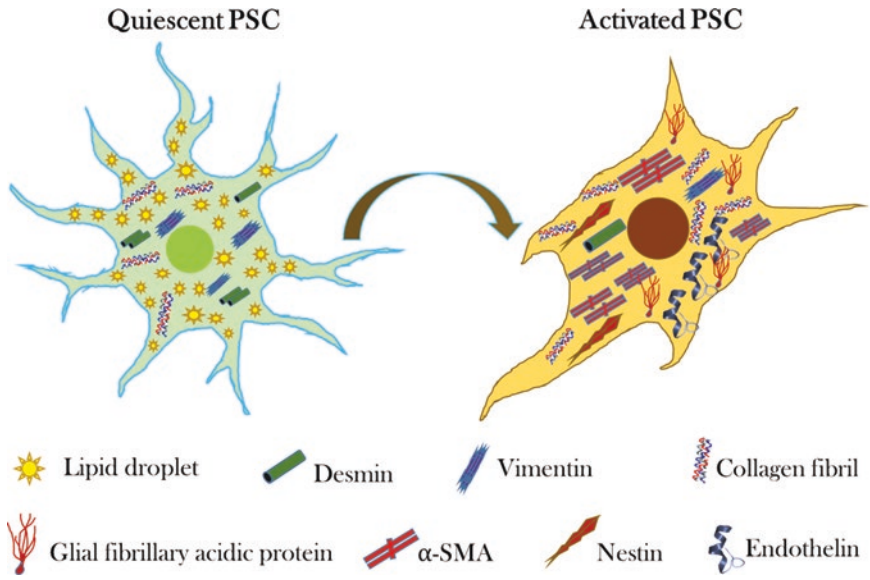

Fig. 5.1 Characteristics of quiescent and activated PSCs. Quiescent PSCs exhibit abundant vitamin-A-containing lipid droplets and ECM proteins such as desmin, vimentin, and collagen fibrils. Activated PSCs exhibited fibroblast-like structure with loss of lipid droplets and increased expressions of α-SMA, endothelin, collagen fibrils, glial fibrillary acidic proteins, nestin, and other ECM proteins

between MMPs and TIMPs; secretion of various cytokines and chemokines; and enhanced migratory and proliferative potential [17, 18]. The characteristics of quiescent and activated PSCs are illustrated in Fig. 5.1.

Presence of vitamin A droplets in the cytoplasm is a consistent marker for the quiescent PSC. Though activated PSCs express abundant α-SMA, it cannot be considered as an exclusive marker as it is expressed by myofibroblasts, smooth muscle cells in the duodenum, blood vessels, pericytes, etc. Expressions of desmin and nestin are also highly variable in PSCs. However, glial fibrillary acidic protein is one of the reliable markers for the activated PSCs as it is absent in the fibroblasts [19]. Activated PSCs attain a spindle-like phenotype resembling fibroblasts, exhibiting enhanced migratory and proliferative potential due to increased production of collagen fibrils and fibronectin [6]. Moreover, the presence of intermediate filament proteins provides specific characteristics to PSC that resemble other cell types. For example, the presence of GFAP provides the characteristics of astrocytes; the presence of desmin resembles myocytes; nestin characterizes neuroepithelial stem cells; and vimentin characterizes fibroblasts and endothelial cells [20]. Presence of such a wide range of intermediate filament proteins provide contractility, with the potential to trigger ECM production and potential to proliferate to PSCs. Unfortunately, however, little is known about the transcriptional regulation, epigenetics, and chromosome dynamics during these phenotypical transitions, which needs further evaluation.

5.3 Stellate Cells: Starring Cells in Pancreatitis, Pancreatic Fibrosis, and Adenocarcinoma

Researches on organ injury by inflammation have proved the pathobiological functions of PSCs to some extent. Though PSCs exhibit various markers that are expressed in stem cells, convincing functional data are not available proving the efficiency of PSCs to transform into another cell

type of pancreas [21]. Still, PSCs are capable of substituting the lost cellular components with fibrotic tissue which is essential for maintaining organ integrity. However, extended activation of PSCs may result in the excessive deposition of matrix proteins which leads to the permanent tissue scarring [22, 23]. In contrast, if the injury and inflammation are limited or governed, PSCs may undergo apoptosis or revert to quiescence. In this way, pancreatic fibrosis is regulated both qualitatively and quantitatively by the persistent activation of PSCs, and therefore it can be considered as the key orchestrator of pancreatic fibrosis.

Multiple studies have shown that oxidative stress, changes in the organization of ECM, and production of cytokines such as interleukins (IL-1, IL-6) and tumor necrosis factor alpha (TNF-α), growth factors such as transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF), and ethanol and its metabolites are the major regulators for the activation of PSCs [24–26]. Macrophages, pancreatic acinar cells, endothelial cells, platelets, and ductal cells in the inflamed pancreas are the major sources of these activating factors. Repeated episodes of acute injury and inflammation activate PSCs surrounding the acinar region. Activated PSCs attract cytokines and chemokines to the site of inflammation. Importantly, activated PSCs also secrete autocrine factors such as cytokines, chemokines, and growth factors that can perpetuate the activated phenotype and thereby play a central role in the inflammatory milieu. The molecular mechanisms triggering pancreatitis remain elusive; however, it is putative that pancreatitis is initiated by injury to the ductal, acinar, and mesenchymal cells in the pancreas [27]. In human and rodent pancreas, activated PSCs are usually found in the areas of extensive injury that further facilitate the production of cytokines and chemokines and create an environment favorable for the inflammatory response [28]. Experimental evidence indicates that ethanol metabolites and reactive oxygen species (ROS)-mediated external insults induced inflammatory response that precedes the activation of PSCs which is prerequisite for its activation [16, 29]. In turn, activated stellate cells enhance cell proliferation and migration and ECM deposition that results in fibrosis. The major events upon the activation of PSCs are illustrated in Fig. 5.2.

Interestingly, TGF-β is known as a notorious factor for the induction of organ fibrosis. Pancreatic acinar cells are the major source for the production of TGF-β in the pancreas. In this way, it is possible that TGF-β produced by acinar cells secondary to injury may be one of the predominant factors behind fibrotic response in PSCs [30, 31]. It has been reported that activated PSCs express membrane type-1 MMP and TIMP-2, and therefore it activates MMP2. Metalloproteases help to degrade the basement membrane which facilitates cell migration [32]. Moreover, in the fibrotic area, α-SMA-expressing cells only encode mRNA for collagen 1α; it is possible that activated PSC is the predominant source for collagen production in the fibrotic area [33]. Most of the available reports suggest the concept that PSCs are activated upon damage to the pancreas, and the inflammatory responses resolve and the activated stellate cells may progressively vanish after the cessation of the injury. However, repeated episodes of chronic injury accompanied by failure in tissue-repairing mechanisms lead to chronic inflammation, persistent activation of PSCs, and finally fibrosis [16, 22, 34]. In fact, organ fibrosis is a consequence of aberrant wound-healing response to chronic injury. Alcoholic consumption, metabolic disorders, genetic defects, and pancreatic duct obstruction are the known causative factors for human pancreatic injury [35]. The chronic injury results in the prolonged activation of PSCs. In addition to the factors discussed here, other signaling pathways are also responsible for the persistent activation of PSCs which will be discussed later.

Extensive desmoplasia is a protuberant feature of PDAC microenvironment. Activated PSCs and cancer-associated fibroblasts (CAFs) are the major constituents of the PDAC stroma which in turn profoundly affect tumor cell behavior [22, 36]. Administration of activated PSCs in orthotopic nude mice resulted in increased tumor formation and metastasis [37] indicating the specific

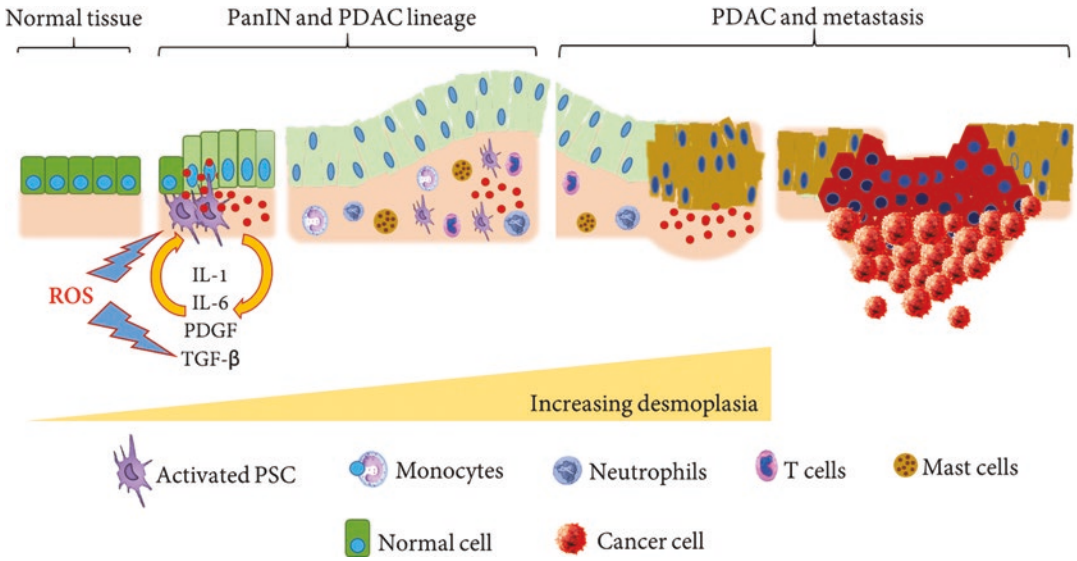

Fig. 5.2 Activated PSC-mediated events in the pancreas. ROS-mediated external insults results in the activation of PSCs by autocrine products such as IL-1, IL-6, PDGF, and TGF-β. Activated stellate cells migrate towards the site of injury and further facilitate the production of autocrine and paracrine products. Persistent activation of PSCs reorganizes ECM and increase desmoplasia

role of PSCs in promoting PDAC progression. Additionally, research evidence supports the symbiotic relationship between cancer cells and PSCs in promoting tumor growth. The culture supernatant of cancer cells stimulate PSCs and enhance the production of ECM [38]. However, the mechanisms by which activated PSCs and the desmoplasia enhance the proliferation of tumor cells are complex and only partly explained.

Dense fibrotic stroma surrounding the tumor is believed to promote tumor cell survival by preventing apoptosis [39, 40]. This can be achieved by the direct interaction of tumor cells with the ECM proteins. The proliferation of tumor cells demand significant structural changes in the microenvironment and other resident cells including increased production of ECM components such as fibronectin and collagen [41]. Activated PSCs and CAFs in the microenvironment are the major drivers for these architectural changes in the microenvironment [42, 43]. Another possible mechanism by which activated PSCs in the tumor microenvironment promote adenocarcinoma cell growth is that tumor cells and PSCs produce more MMPs and other tissue serine proteases that degrade ECM proteins and basement membrane which allow tumor cells to migrate, invade, and metastasize, as has been postulated in other tumors [44].

5.4 Molecular Signaling Cascades Involved in Pancreatic Stellate Cell-Mediated Desmoplasia

PSCs are the major source of secretory proteins in the tumor microenvironment. Great varieties of cytokines, chemokines, growth factors, exosomes, and other soluble bodies are secreted by the activated PSCs that act either in autocrine or paracrine manner in orchestrating the signal transduction between stroma and tumor cells [45, 46]. The major molecular signaling pathways involved in PSC-mediated desmoplasia are as described below:

5.4.1 Transforming Growth Factor-β/Smad Signaling

TGF-β is a well-known pro-fibrotic signaling mediator involved in the tumor-stromal cross-talk. Research evidence shows that a great amount of TGF-β is produced in the stroma by activated PSCs [47, 48]. In the classic signaling pathway, latent TGF-β interacts with a cytoplasmic receptor in activated PSCs and phosphorylates its canonical downstream signaling molecule Smad2/3. Phosphorylated Smad2/3 oligomerizes with Samd4 and translocates to the PSC nucleus. Through interaction with a variety of transcription cofactors, it induces the transcription of ECM proteins especially collagen 1 which further promotes desmoplasia in PDAC [30, 49]. However, the functions of TGF-β vary depending on the tumor microenvironment. In a non-cancerous epithelium, TGF-β acts as a tumor suppressor, whereas in a cancerous cell, TGF-β promotes cell proliferation, migration, and tumor metastasis that have been associated with epithelial-to-mesenchymal transition (EMT) process [50]. Co-culturing of PDAC cells with PSCs exhibited elongated fibroblast-like morphology; decreased expression of E-cadherin, cytokeratin 19, and membrane-associated β-catenin along with increased expressions of vimentin and snail than mono-cultured PDAC cells indicates the potential role of PSCs in inducing EMT in cancer cells [51]. Along with TGF-β, several other cytokines and proteins such as connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), and periostin secreted by PSC are involved in the induction of EMT [52–54].

PSC-derived TGF-β in the stroma is also responsible for chemoresistance in PDAC. Of the various cells in PDAC stroma, PSCs and CAFs are the major fibrosis-inducing cells [55, 56]. Activated PSCs are known as the master secretors of soluble and insoluble factors that specifically form dense stroma surrounding the tumor which outnumber the tumor cells. Genetic variations of TGF-β promote PDAC tumor progression and chemoresistance pointing out the decisive role of PSC-derived TGF-β in inducing chemoresistance in PDAC [57]. Interestingly, reports show that TGF-β further stimulates chemotherapy-resistant subpopulation of cells to undergo EMT which makes it more aggressive, invasive, and highly resistant to chemotherapeutic drugs [58].

5.4.2 Hedgehog Signaling

A hedgehog signaling pathway is another important signaling in PSCs. Inhibition of Sonic Hedgehog (SHH) signaling decreased desmoplasia, and its overexpression promoted the formation of dense fibrotic stroma supporting the concept that SHH is an important signaling cascade during PDAC progression [36]. Moreover, it has been reported that Indian Hedgehog (IHH), another member of hedgehog signaling, promotes activated PSC migration through the localization of type 1 MMP on the cell surface [59]. Jennifer and colleagues identified that SHH actively induces the differentiation of PSCs into myofibroblasts [36]. They have stimulated PSCs with recombinant SHH (1 and 10 μg/ml) for 24 h and found an increase in the expression of mesenchymal markers concomitant with a decrease in the expression of epithelial markers. This was the first study indicating the potential of PSCs to differentiate into another phenotype [36]. Yet another interesting observation is that the ligands for the oncogenic allele of Smoothened (SmoM2) which autonomously activate hedgehog signaling is observed in stromal-derived PSCs only but is limited to tumor cells [60].

5.4.3 Wnt/β-Catenin Signaling

Another important signaling pathway whose aberration could result in the activation, proliferation, and transformation of PSCs into fibrotic phenotype is Wnt/β-catenin signaling. Two different pathways have been described: canonical Wnt signaling and noncanonical Wnt signaling. Stimulation of the canonical Wnt signaling results in the accumulation and nuclear translo-

cation of β-catenin that mediate cellular processes in response to Wnt [61]. Research evidence supports that PSC activation may depend on Wnt signaling activation and the imbalance of Wnt/Dickkopf protein families (Dkks), which negatively modulate the canonical Wnt pathway promoting the persistent activation of PSCs [62]. They have further provided evidence that inhibition of Wnt signaling using the antagonist Dkk significantly inhibited PSCs activation and collagen synthesis by downregulating the expressions of TGF-β receptor II and PDGF receptor β [62]. Yet another study revealed that co-culture of PSCs with cancer cells activates the classical Wnt signaling pathway in PDAC cells [63]. In support of this observation, Xu et al. have demonstrated that Wnt 2 protein in the stroma may activate PSCs which further stimulate the activation of canonical Wnt/β-catenin signaling cascade in PDAC cells [64].

5.4.4 Mitogen-Activated Protein Kinase (MAPK) Signaling

Mitogen-activated protein kinase (MAPK) signaling cascade includes three different families of serine-threonine protein kinases; p38, extracellular signal-regulated kinase (ERKs), and c-Jun N-terminal kinase (JNK) [65]. All these three MAPKs have been extensively studied for their role in the activation of PSCs. Research evidence has demonstrated that initial activation of ERK1/2 precedes the transformation of PSCs from a quiescent state to an activated phenotype. ERK-specific inhibitor significantly suppresses the growth of PSCs revealing the importance of ERK signaling during PSC activation and differentiation [66]. Yet another research group has reported that all three MPAK are involved in the activation of PSCs when stimulated with ethanol or aldehyde through the activation of activator protein-1 [25]. In addition to this, the treatment of PSCs with a specific inhibitor of p38 MAPK significantly inhibited the expression of α-SMA by PSCs [67].

5.5 Stellate Cell-Cancer Cell-Stromal Interaction in the Pancreas

It has been conclusively proven that 80% of the PDAC volume is composed of desmoplastic stroma, and cumulating evidences substantially corroborate the two-way interactions between tumor cells and stromal components [68–70]. Desmoplastic stroma in the PDAC is predominantly composed of fibrous components laid down by PSCs along with cellular components such as lymphocytes, endothelial cells, and mast cells; non-cellular ECM proteins such as collagen, elastin, fibronectin, and laminin; and non-ECM components such as stellate or cancer cell-derived growth factors [71–73]. The stromal components mediate the interactions between PSCs and tumor cells and influence tumor cells' biological behavior and eventually promote PDAC progression. This hypothesis was substantiated in an orthotopic mouse model where mice co-administered with PSCs and PDAC cells were exhibited and enhanced local and distant metastatic tumors relative to only injecting PDAC cells [37]. Co-culturing of PDAC cells with PSCs facilitate tumor migration through the induction of EMT [51]. In addition, culture supernatant of PSCs promoted proliferation, invasion, migration, and chemoresistance of cancer cells [71, 74] also supporting the hypothesis that PSCs interact with cancer cells and providing an aggressive behavior for the tumor progression.

Since fibrosis is an early event to PDAC development, initially it was believed that PSC-derived stroma is protective against the tumor progression. However, the opinion is eventually shifted towards the concept that stellate cell-stromal-cancer cell interactions are dynamic, stage and context dependent which may be protective at the earliest stage, however obviously harmful at the later stage [75]. Evidence showed that two-way interactions between PSCs and cancer cells that significantly influence each other are essential for tumor growth. For instance, PDAC cells produce

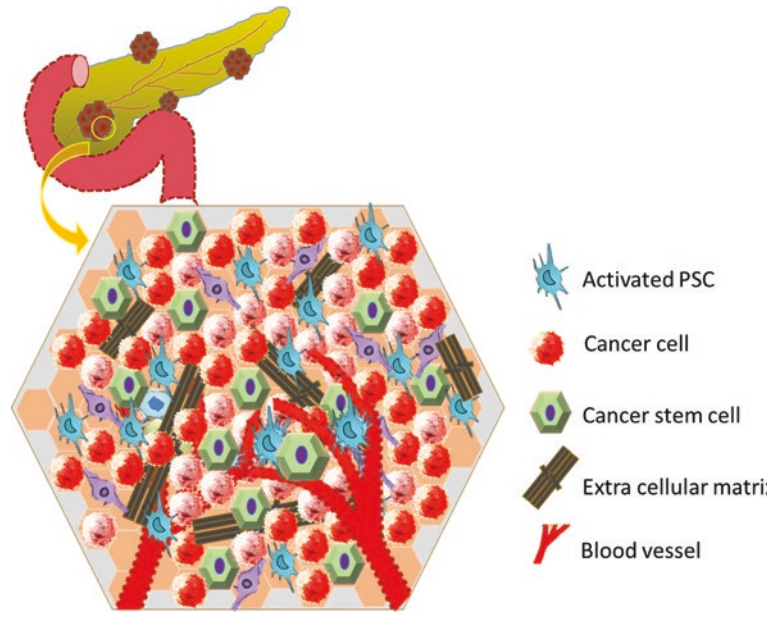

Fig. 5.3 Interactions between PSCs, stroma, and tumor cells in the microenvironment. PSCs are instrumental in the tumor microenvironment promoting tumor cell migration, invasion, EMT, and metastasis. PSC-derived stroma provides a favorable niche for the tumor cells to proliferate

factors such as PDGF, TGF-β, cytokines, and chemokines and COX-2 could induce the proliferation of PSCs [76, 77]. In return, PSCs produced growth factors that enhance tumor growth and MMPs degrade the basement membrane which facilitates tumor cell migration and invasion [78, 79]. Interactions between PSCs with stromal cells are considered as instrumental in tumor metastasis, invasion, and chemoresistance. PSC-mediated stroma in the tumor microenvironment is outlined in Fig. 5.3.

5.6 Therapeutic Implications of Pancreatic Stellate Cells

Due to the central and decisive role of PSCs in the PDAC desmoplasia, these are deliberated as an attractive target for treatment. Several experimental studies that targeted pro-fibrogenic PSCs have shown favorable results in regulating PDAC progression and metastasis. For instance, Sherman et al. have reported that vitamin D receptor (VDR) ligand calcipotriol significantly reduced fibrotic stroma specifically through the transcriptional regulation of PSCs to reprise the quiescent state [80]. Another study has found that retinoic acid-induced quiescence in PSCs reduced tumor cell proliferation through regulating Wnt signaling [81]. Based on the immunosuppressive role of activated PSCs that regulate T-cell migration, alteration in PSC function was found as an effective mode to restore anti-tumor response [82]. Since PDAC stroma has been found to be associated with hypoxia and drug resistance, drugs that degrade stroma are expected with good clinical outcome [83, 84, 85]. Therapeutic agents that specifically target PSCs have been summarized in Table 5.1.

5.7 Conclusion and Future Perspectives

Extensive desmoplasia is a unique characteristic of pancreatic ductal adenocarcinoma. PSCs are considered as the foremost active player in the induction of PDAC desmoplasia. Though much more remains to be elucidated about the biological role of PSCs in PDAC, understanding of their functions, transition from quiescent to active state, and crosstalk with tumor cells and stroma are expected to pave the way in the fight against PDAC progression. Considering the dual role of stroma in PDAC, stromal reprogramming targeting PSCs rather than depletion may open new

Table 5.1 Therapeutic agents targeting pancreatic stellate cells against PDAC progression

Agent	Target/type	Outcome of the study	References
AdTbeta	PSC-derived TGFβ	Reduction of activated PSCs, decreased pancreatic fibrosis, prevented acinar cell apoptosis	[86]
Allopurinol	PSC activation	Inhibited PSC activation, reduced pancreatic fibrosis through xanthine oxidase metabolism	[87]
Bisphosphonates nab-paclitaxel	Osteoclast inhibitor	Inhibited PSC proliferation, activation, release of MCP-1, and synthesis of type I collagen. Induced PSCs apoptosis	[83]
Bosentan	Endothelin receptor antagonist	Inhibited cancer cell proliferation and collagen synthesis in PSC. Reduced chronic pancreatitis	[88]
Bone morphogenetic proteins	TGFβ in PSCs	Inhibited TGF-β induced α-SMA, collagen and fibronectin in PSCs	[89]
Camostat mesilate	Protease inhibitor	Inhibited inflammation, cytokine expression, and fibrosis by inhibiting monocyte and PSCs activity	[90]
Cannabinoid	Cannabinoid receptors on PSCs	Induced deactivation of PSCs, decreased IL-6 and MCP-1 secretion, fibronectin, collagen 1, and α-SMA	[91]
Carbon monoxide-releasing molecule-2	P38-MAPK	Inhibited PSC proliferation and activation	[92]
Eruberin A	Flavanol glycoside	Suppressed the expressions of type 1 collagen, α-SMA, and fibronectin in PSCs through the regulation of Sonic Hedgehog signaling pathway	[93]
EGCG	Green tea poly-phenol	Suppressed p38-MAPK phosphorylation, α-SMA production, and TGF-β secretion in PSCs	[94]
Interferon β/γ	Cytokine	Decreased PSC activation, proliferation, and collagen synthesis	[95]
L49H37	Curcumin analog	Inhibited PSC proliferation and induced apoptosis of PSCs through the regulation of ERK signaling	[96]
Octreotide	Growth hormone inhibitor	Inhibited α-SMA and collagen 1 synthesis of PSCs	[97]
Prostaglandin E2	Prostaglandin	Suppressed the proliferation of PSCs, inhibited the formation of fibrotic stroma	[85]
1,25-dihydroxyvitamin D3	Vitamin D metabolite	Reduced fibronectin and collagen 1 expressions in PSCs	[98]
Trametinib and dactolisib	Small molecule kinase inhibitors	Reduced PSC proliferation by specifically targeting Ras-Raf-MEK-ERK (trametinib) and PI3-kinase-AKT-mTOR (dactolisib) signaling	[99]
Y-27632 and HA-1077	Rho kinase inhibitor	Significantly decreased PSCs activity and collagen production by regulating actin cytoskeleton	[100]

avenues for translational medicine and better clinical therapies for PDAC.

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Endothelial Cells in the Tumor Microenvironment

6

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Abstract

Angiogenesis is a critical process required for tumor progression. Newly formed blood vessels provide nutrition and oxygen to the tumor contributing to its growth and development. However, endothelium also plays other functions that promote tumor metastasis. It is involved in intravasation, which allows invasive cancer cells to translocate into the blood vessel lumen. This phenomenon is an important stage for cancer metastasis. Besides direct association with cancer development, endothelial cells are one of the main sources of cancer-associated fibroblasts (CAFs). The heterogeneous group of CAFs is the main inductor of migration and invasion abilities of cancer cells. Therefore, the endothelium is also indirectly responsible for metastasis. Considering the above, the endothelium is one of the important targets of anticancer therapy. In the chapter, we will present mechanisms regulating endothelial function, dependent on cancer and cancer niche cells. We will focus on possibilities of suppressing pro-metastatic endothelial functions, applied in anti-cancer therapies.

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Keywords

Endothelial cells · Cancer development · Tumor endothelial cells · Cancer microenvironment · Cancer niche · Sprouting · Metastasis · CAFs · Microvessels · Tip cells · Tumor angiogenesis · VEGF · Hypoxia · Endothelial-mesenchymal transition · TGF-β

6.1 Introduction

The vascular endothelium is a versatile structure that separates the circulating blood from tissues. Moreover, apart from regulation and maintenance of blood fluidity, it plays multifunctional roles in the delivery of water and nutrient, maintenance of metabolic homeostasis, trafficking of immune cells, activation of innate and acquired immune responses, as well as angiogenesis [30, 73]. The endothelium is a thin monolayer, composed of endothelial cells (ECs) that are able to organize the growth and development of connective tissue cells, forming the surrounding layers of the blood vessel wall. This process is controlled by a paracrine/endocrine network which involves fibrinolytic, pro- and anticoagulants, vasoactive, pro- and anti-inflammatory factors, as well as growth factors produced by ECs [84]. Thus, ECs must be constantly poised to sense and respond to changes within their environment. In tumor and its microenvironment, some agents

A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_6

like hypoxia and chronic growth factor stimulation might lead to endothelial dysfunction. There is more and more evidence showing that these abnormalities contribute to cancer progression.

The tumor has been recently described as an aberrant organ not only composed of cancer cells but also of numerous stromal, inflammatory, and vascular cells. Like other organs, in order to develop, the tumor requires a blood supply to provide nutrients and oxygen and waste removal. Initially, cancer cells might adopt tissue-resident vessels. However, the tumor eventually recruits its own vascular supply through the angiogenesis process [123]. The tumor-associated angiogenesis has been defined as sprouting of new vessels from preexisting vessels, which involves endothelial cells [112]. Tumor modulates its microenvironment by releasing numerous cytokines, chemokines, and growth factors to activate normal, quiescent endothelial cells and adapt them to the angiogenic response. Moreover, surrounding stromal cells might also secrete a plethora of factors and cytokines influencing tumorigenesis and metastasis. Within them, TGF-β is considered one of the main factors modulating interactions between cancer and surrounding cells, located within the tumor niche. Among the TGF-β-dependent effects is regulation of cancer cell proliferation, affecting immune response by suppressing immune cells function, conversion of fibroblasts to myofibroblasts and epithelial-mesenchymal transition (EMT). Furthermore, TGF-β promotes the formation of cancer-associated fibroblasts (CAFs), a specialized group of fibroblasts involved in tumor growth and invasion of cancer cells by modulation of the tumor niche [119]. Until now normal fibroblasts (NFs) have been considered the main source of CAFs, but in the last years, endothelial cells have also become an important origin of CAFs. It has been shown that TGF-β is responsible for such EC conversion in a process called endothelial-mesenchymal transition (EndMT) [56]. During EndMT, endothelial cells lose endothelial markers and gain mesenchymal ones, which is followed by increased expression of transcription factors such as Snail and Slug. The changes are accompanied by defaulting of their cellular function and taking on some characteristics of mesenchymal cells, including loss ability to form capillary tubes and cell-cell junctions, increased cell migration properties, and secretion of extracellular matrix proteins.

In this review, we will focus on the role of endothelial cells in tumor microenvironment particularly on their direct and indirect role in cancer metastasis. While endothelial cells were originally believed to be involved in the direct development of primary tumor due to vascularization, there is more and more evidence suggesting their indirect effect on cancer progression. CAFs are known to play an important role in tumor growth and progression via secretion of various growth factors and chemokines. The contribution of endothelial cells in CAF formation will be discussed. Finally, we will also present current and future therapeutic possibilities targeting at endothelial cells, CAF formation, and chemokines in the context of anti-metastatic treatment.

6.2 Heterogeneity of Normal and Tumor Endothelial Cells

The vascular endothelium is a specific inner cellular lining that separates the circulating blood from the tissues. That thin monolayer plays an important multifunctional property, including the control of vasomotor tone, proliferation/angiogenesis, permeability, hemostasis, humidification, thermoregulation, leukocyte transmigration, sieve function, and scavenging innate and adaptive immunity [2]. This plethora of functions is a consequence of the fact that ECs, being part of the vascular tree, are differentially regulated in space and time. Thus, ECs differ in various organs, but also between distinct segments within or between neighboring of vascular architecture of the same organ. The EC thickness varies across the vascular tree, ranging from less than 0.1 μm in capillaries and veins to 1 μm in the aorta [2]. Endothelial cells are usually flat, but they might be plump or cuboidal occasionally [2]. Endothelium cells in monolayer are held by two main types of junctions: adherent junctions (AJs) and tight junctions (TJs). Their organization

varies along the vascular tree [12]. For instance, the large artery is rich in TJs, whereas venules display less organized TJs. Similarly, in the brain, where protection of the nervous system is required, junctions are well developed and rich in TJs [31]. In contrast, post-capillary venules have a poorly organized TJs due to the dynamic trafficking of circulating cells and proteins suspended in plasma [31]. Another feature of endothelium diversity is its continuity. Continuous endothelium might be fenestrated or non-fenestrated. Fenestrated continuous endothelium is found in the places where increased filtration or increased transendothelial transport is needed, like capillaries of exocrine and endocrine glands. Non-fenestrated continuous endothelium is found in capillaries, veins, and arteries. Discontinuous endothelium occurs in some sinusoidal vascular beds, first of all in the liver [2]. It has been proposed that angiogenesis, being one of the main processes engaging endothelial cells, requires at least a few cells of discontinuous. ECs, called tip cells, are directly engaged in vessel sprouting. Highly proliferative stalk cells follow tip cells, and phalanx cells that are involved in improving the perfusion and oxygenation of newly formed blood vessels [51].

Mentioned ECs heterogeneity is provided mainly by one of two distinct mechanisms based on microenvironment pressure or epigenetic modulation [3]. Endothelium is not only a specific inner cellular lining separating the circulating blood from the tissues, but it is exposed to a great variety of factors, secreted by tissue microenvironments. Moreover, to properly perform its functions across the vascular tree, ECs have to detect and respond to environmental stimuli, which is guaranteed by endothelial cells heterogeneity. This mechanism is reversible when ECs are removed from their microenvironment and grow in tissue culture. The second mechanism involved posttranscriptional modification that seemed to be epigenetically programmed and independent of extracellular signals. Although it is widely accepted that microenvironment stimulation is responsible for triggering epigenetic modifications, they may remain during the removal of the signals and be transmitted during mitosis [3].

It should be noted that EC heterogeneity also translates into the heterogeneity of tumor endothelium. In line with Folkman's hypothesis, tumor growth strictly depends on blood vessels [41]. At the same time, tumor blood vessels are formed by ECs recruited from surrounding tissue transformed to tumor endothelial cells (TECs). The tumor vasculature, in contrast to well-differentiated normal vessels, it is composed of a chaotic mixture of abnormal, disorganized artery–capillary–vein hierarchy vessels [109]. Unlike normal blood vessels, tumor vessels are more dilated and tortuous. They branch irregularly, have chaotic flow patterns, and increased permeability to macromolecules [75]. Due to an imbalance between pro- and antiangiogenic factors and with a predominance of stimulators (angiogenic switch), a classic hierarchical branching pattern system of arterioles, veins, and capillaries is disturbed. The layout of neoplastic capillaries is morphologically immature: chaotic, strongly twisted, with variable vessel diameter and irregular edge [29]. In line to the unsettle tumor vasculature, endothelial cells, forming tumor vessels, are structurally abnormal. TECs have a disturbed redistribution of phospholipids, a discontinuous or absent basement membrane, increased fenestrations and extended intercellular junctions, and a high proliferative rate compared to normal ECs and tend to grow one on top of the other and invade into the vessel lumen [3]. Phenotypic changes, accompanied by changes at the molecular levels, have been identified comparing normal ECs to TECs, isolated from normal and tumor tissues. In 2000, St. Croix et al. performed a comparative analysis of gene expression profiles between tumor endothelial cells and normal endothelial cells and identified the specific genes for TEC called tumor endothelial markers (TEMs) [95]. Since then, several studies have been published on molecular differences between TECs and NECs [15, 66, 77] e.g tumor endothelial markers (TEMs), endoglin (CD105), or endothelial protein-disulfide isomerase EndoPDI [50] has been also demonstrated that TECs can secrete several factors that affect their survival in an autocrine manner [17, 18, 74, 101].

Increased permeability of the walls, hemorrhage, and plasma leakage result from a reduced number of pericytes and increased proteolytic activity within the vessel formation zone. TECs are characterized not only by an increased size, but they also presented aneuploidy, abnormal centrosomes, and high activation of the MAPK pathway, promoting cell survival [5, 43]. TECs exhibit several differences which contribute to their proangiogenic phenotype, including changed responsiveness to growth factors such as EGF, adrenomedullin, and VEGF. VEGF stimulates the migration of TECs and enhances their survival in an autocrine manner, which leads to the antiapoptotic phenotype of TECs [51]. TECs show upregulated aldehyde dehydrogenase (ALDH) expression which is manifested by a formation of increased tube number even under starvation conditions [80].

It is suggested that the persisting hypoxia together with the secretion of cytokines promotes tumor angiogenesis by inducing the mobilization of bone marrow-derived endothelial progenitor cells to cancer [45]. Glioblastoma cells and lymphoma ones are examples of tumor cells that are capable of differentiating into TECs [98, 106]. Interaction between tumor cells and the microenvironment leads to alteration of ECs into TECs that express high levels of biglycan through epigenetic modifications, which stimulates tumor cells to metastasize through activation of different signaling pathways [67]. Furthermore, it was reported that endothelial progenitor cells release microvesicles with gene fragments that can activate endothelial cell angiogenic properties [33]. Due to the mechanisms mentioned above, TECs become cytogenetically abnormal and unstable in the tumor microenvironment.

6.3 Angiogenesis in Tumor Development

Efficient functioning of the circulatory system, responsible for gas exchange, transport of nutrients, and metabolic products, is the basic condition for appropriate development during ontogeny. In embryo development, de novo formation of the vascular plexus from angioblasts (EPCs; endothelial precursor cells) is one of the earliest organogenesis processes, called vasculogenesis [1]. Next, the existing vascular network undergoes proliferation, reorganization, and maturation in the process of angiogenesis (neovascularization) [11]. A new capillary mesh network is created by sprouting of endothelial cells. The last stage is the maturation of the vessel through the migration of pericytes and vascular smooth muscle cells (VSMCs) on a newly formed basal membrane (BM). Under physiological conditions, neovascularization occurs during embryo implantation, the women's monthly cycle, and wound healing, and in the muscles [16]. In pathological conditions, when the activity between pro-angiogenic factors and antiangiogenic ones is disturbed, it occurs during chronic inflammation and hypoxia and in asthma, rheumatoid arthritis, psoriasis, Crohn's disease, diabetic retinopathy, as well as endometriosis and obesity. However, angiogenesis plays the most significant role in the process of neoplasia [65].

In the initial stage, in order to survive and proliferate, tumor takes oxygen and nutrients by diffusion. The environment in which it develops undergoes hypoxia and acidification as a result of excess metabolic products. When its volume exceeds 1–2 mm^3, the tumor must become angiogenic and recruit their vasculature to grow. Cancer cells, together with host/niche cells, stimulate the development of their blood vessels, using various mechanisms of tumor angiogenesis [36]. The most common one and best described is vessel sprouting (Fig. 6.1). In the classical model, the vasodilatation of the mother vessel occurs, which contributes to reduced BM density. It leads to partial degradation of BM and protrusion of endothelial cells in that place. As the ECs do not lose intracellular connection with each other and they migrate parallelly, the polarity of the cells is preserved. At the same time, the new lumen is formed by polarized ECs. They release proteins which rebuild the basal membrane along which pericytes migrate. This phenomenon stabilizes the capillary and contributes to its maturation [83]. The last step of vessels maturation described above is impaired during cancer angiogenesis.

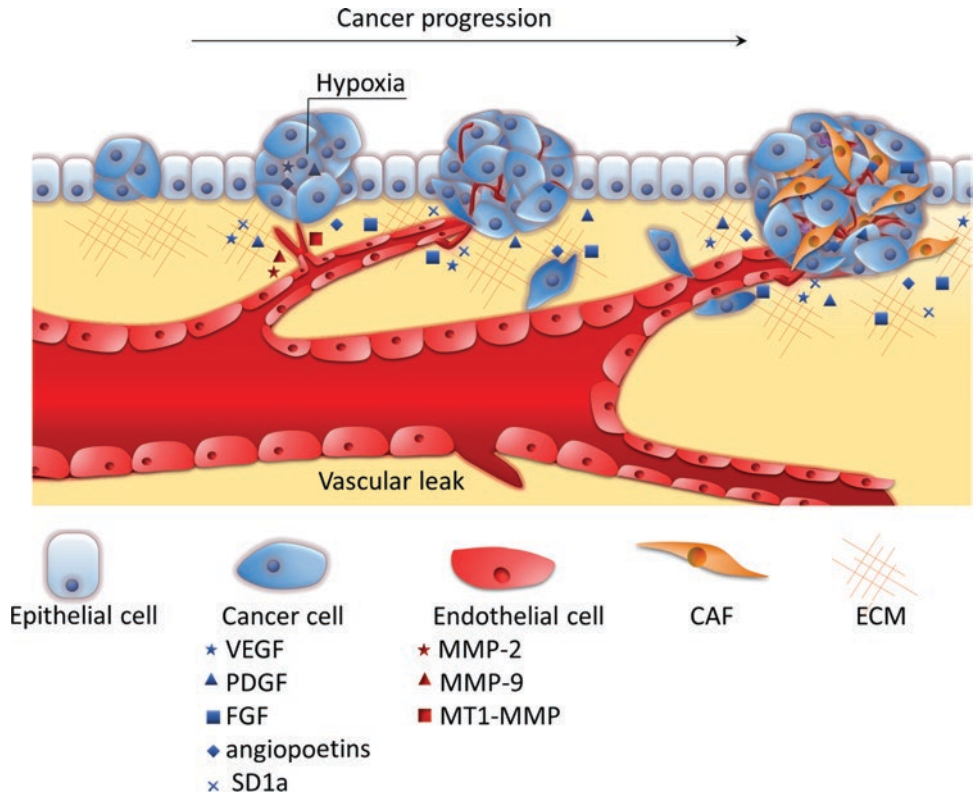

Fig. 6.1 Mechanisms of tumor vascularization. At the point when developing cancer reaches its size 1–2 mm, hypoxia and nutrient deprivation result in release of tumor cell-soluble growth factors, chemokines, and cytokines (VEGF (blue star), PDGF (triangle), FGF (square), angiopoietins (diamond), and SD1a (cross)). The factors induce the sprouting and proliferation of endothelial cells on nearby blood microvessels. The created tumor blood vessels are leaky and tortuous with partially exposed basal lamina where vascular leaks are observed. Additionally, the vascular remodeling is also enhanced by factor secreted by cancer-associated fibroblasts (CAFs) that are recruited to the tumor niche. CAFs cause the rearrangement the profile of extracellular matrix protein and release matrix metalloproteinases (MMPs: MMP-2 (red star), MMP-9 (triangle), and MT1-MMP (square)) that cleave and remodel ECM therefore activating the endogenous angiogenesis inhibitors such as tumstatin and endostatin

Hypoxia-induced factor-1α (HIF-1α) is the main factor that initiates sprouting [69]. It induces secretion by ECs proangiogenic factors such as platelet-derived growth factor, type B (PDGF-B), hepatocyte growth factor (HGF), angiopoietins, epidermal growth factor (EGF), placental growth factor (PlGF) [29, 65] and is the main stimulator of angiogenesis vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) [94]. VEGF, which works in an auto- and paracrine manner, contributes to extravasations of plasma proteins, e.g., fibrinogen, which initiates integrin-dependent migration of ECs, a release of metalloproteinases, and activation of the mitogen-activated protein kinase (MAPK) pathway. Digestion by MMPs of extracellular matrix (ECM) releases the tumor growth factor (TGF-β), basic fibroblast growth factor (FGF-2), and insulin-like growth factor-1 (IGF-1), i.e., anti-apoptotic factors, activating the survival signal transduction pathway [27, 47, 52]. The second group consists of tissue-resident cells, including normal tissue epithelial cells, vascular cells (endothelium and pericytes), normal fibroblasts, adipocytes, and leukocytes (mast cells and macrophages) [20, 22, 34, 92]. It has been confirmed that leukocytes as well as ECs are important sources of VEGF-A, which is able to accelerate tumor angiogenesis [35]. But TASCs might increase vascular density in human tumors

through secretion of other numerous chemokines and growth factors (Fig. 6.1).

Cancer stem cells (CSCs) can differentiate to endothelial cells and, as a consequence, induce new vessels via a phenomenon known as vascular mimicry. However, that ability does not lead to the form of mature and proper blood vessels which would counteract hypoxia. During progression, cancer recruits numerous types of cells to the cancer niche, which can modulate tumor vascularization. The cells located in the tumor microenvironment, called tumor-associated stromal cells (TASCs), can be divided into two main groups. Leukocytes (lymphocytes, neutrophils, monocytes, and macrophages) infiltrating tumor constitute the first group delivered from the bone marrow via systemic circulation. Macrophages that are recruited to the tumor environments, called TAMs (tumor-associated macrophages), have been described as a source of non-thrombogenic EC-like surfaces, constituting a potential scaffolding for tumor vascularization through mimicry vasculare [89]. However, the mechanism of that process is still unknown.

Tumor cells play a crucial role in initiation and regulation of cancer angiogenesis. It must be noted that, other cells, located in the tumor niche, also secrete numerous signaling molecules and induce pathways that influence the angiogenic response. Apart from sprouting new vessels in response to VEGF stimulation, blood vessels might also originate from cells of the bone marrow or tumor stem cells dedifferentiated to ECs (vascular mimicry). A wide diversity of molecular pathways which are able to induce tumor vascularization can make antiangiogenic therapies ineffective [96].

Tumor endothelial cells may undergo endothelial to mesenchymal transition (EndMT) and become carcinoma-associated fibroblasts, CAFs. It was demonstrated that stromal-derived factor-1 (SDF-1) in CAFs recruits EPCs promoting angiogenesis. Overexpression of MMP-2 by CAFs stimulates epithelial hyperplasia and abnormal branching in the mammary gland. It was shown that high level of MMP-2 production in stromal cells is required to support pathological neoangiogenesis of gliomas. Neovascularization is promoted also by induction of IL-8 secretion by CAFs, isolated from metastatic colon cancer patients [117]. CAFs express a membrane-bound serine protease, called fibroblast activation protein (FAP), which is associated with poor prognosis in several cancer types.

Significant associations were found between tumor angiogenesis and miRNAs in activated endothelial cells. miRNAs have opposing effects on cancer and endothelial cells. Their overexpression inhibits angiogenesis and enhances proliferation of cancer cells. MicroRNA-126 (miR-126) is an endothelial-specific miRNA that regulates angiogenic signaling and vascular integrity as a negative regulator of VEGF-A. However, it was observed that overexpression of miR-126 in endothelial cells enhances VEGF-A activity and promotes vessel formation by repressing the expression of sprouty-related protein-1 (Spred-1) [105]. In oral squamous cell carcinoma, a low miR-126 expression is correlated with tumor progression through the activation of angiogenesis and lymphangiogenesis via VEGF-A pathway [91]. miR-126 is involved in cancer cell–stromal cell crosstalk. CAFs induces downregulation of miR-126 in adjacent human umbilical endothelial cells (HUVEC). The lowered miR-126 confers increased tube formation in the early invasive stage of cervical cancer [53]. VEGFR2 can be targeted by miR-221 and miR-222 [55].

6.4 Intravasation of Cancer Cells

Metastasis is a multi-step process, divided into two main phases: (1) translocation of cancer cells from the primary tumor to distant tissues and (2) colonization of these cancer cells at the secondary site [48]. Here we focused on the role of the endothelium in the first phase. The tumor metastatic potential is dependent on its rapid extravasation into the vascular system [13, 61, 82]. That process is composed of several steps: adhesion of invading cancer cells to ECs, changes in the endothelial barrier and intravasation,

dissemination into the bloodstream as migrated and proliferated circulating tumor cells (CTCs), and finally, after extravasation, colonization of other organs [6, 59, 61, 97]. Transendothelial migration (TEM) of invasive cancer is a critical phenomenon in the intra- and extravasation. During that phenomenon, tumor cells migrate between two endothelial cells [61, 93]. In vitro studies suggest that tumor cells might also pass through individual endothelial cells, in a process called transcellular migration [58, 99]. An interaction between transmigrated cancer cells and ECs induces contraction and disruption of their cell-cell contacts as well as secretion of pro-inflammatory factors by the latter [103]. Metastatic microenvironment is also characterized by platelet aggregation and formed microthrombi which promote ECs activation through induced inflammation [100]. As described above, blood vessels arising during cancer progression [49] are usually immature without proper junctional contact between ECs. The blood vessels are leaky and vulnerable due to abnormal pericyte coverage. Those injuries enable cancer cells to intravasate through the blood barrier [37, 116]. According to a favorable theory, both intravasation and extravasation are active processes, regulated by several factors such as TGF-β [7], VEGF [38, 60, 86], angiopoietin-2 (Angpt2) [88], stromal-derived factor-1α (SDF-1α) [118], or TNF [121]. A notable difference between intravasation and extravasation is found in the fact that intravasation mostly involves abnormal tumor vasculature whereas extravasation targets at normal blood vessels. It has been observed that the interaction between tumor cells and ECs is modulated by VEGF or TNF favor intravasation. The process is modulated when the number of blood microvessels increases and disruption of the blood barrier occurs [Fig. 6.2]. Additionally, presence of macrophages seems to be necessary for this process. However, macrophage-secreted TNF increases endothelial permeability, but its depletion does not reduce intravasation. The authors suggested the importance of other macrophage-secreted factors (probably IL-6) or juxtacrine interactions in induction of intravasation. They also prove the importance of remodeling of the endothelial barrier, induced by tumor-endothelial interaction for translocation of tumor cells via the blood barrier [121].

Some data suggested that endothelial-mesenchymal transition, leading to disruption of the cell-cell junction between ECs and disruption of blood barrier, also contributes and facilitates cancer cell intravasation.

6.5 CAF Formation

Endothelial cells forming a single-cell layer lining the inner surface of the blood vessels [55] are characterized by wide plasticity [23]. During cancer progression, the endothelium that undergoes endothelial-mesenchymal transition (EndMT) is becoming, besides normal fibroblasts (NFs), one of the main sources of cancer-associated fibroblasts (CAFs). It has been postulated that about 40% of CAFs are formed from endothelial cells [56].

During EndMT, cells lose cell-cell connections, detach from the cell layer, and elongate. Additionally, their adhesion ability is decreased, and migration properties increased (Fig. 6.3). Those behavioral modulations are accompanied by decreased endothelial marker levels, such as CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)) or claudin, and gain of mesenchymal markers, such as fibroblast-specific protein 1 (FSP1; S100A4) or α-smooth muscle actin (αSMA) [9, 10, 76, 85, 120]. CAFs are also characterized by increased expression of contraction proteins like caldesmon and tropomyosin [26, 124].

The best-known inductors of EndMT belong to the transforming growth factor superfamily (TGF-β) which includes TGF-β1, TGF-β2, and bone morphogenetic protein (BMP). Activation of receptors for these factors leads to an induction of Smad-dependent and Smad-independent pathways [40, 102, 71]. In the canonical pathway, TGF-β1 or TGF-β2 binds to constitutively activate II TGF-β receptor (TGF-βRII) and then to recruit and activate I TGF-β receptor (TGF-βRI)

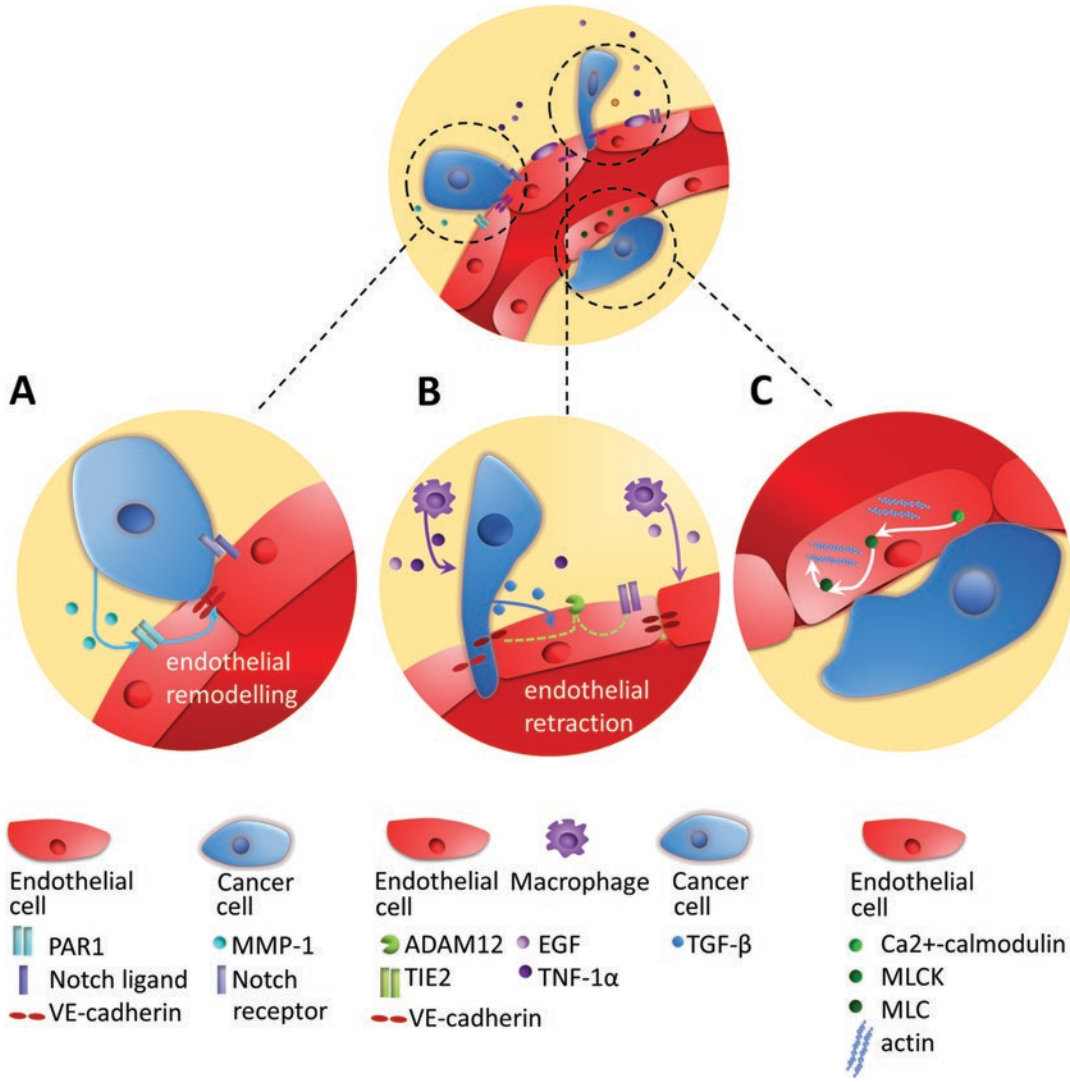

Fig. 6.2 Molecular pathways that regulate the intravasation. Cancer cells enter the circulation by transmigrating either paracellularly through the endothelial cell (EC) junctions or transcellularly through the EC body. Matrix metalloproteinase 1 (MMP-1) is crucial for paracellular intravasation in regions where protease-activated receptor 1 (PAR1) on ECs mediates the remodeling of endothelial junctions (**a**). Cancer cells can use Notch receptors to bind to Notch ligands on ECs and thereby transmigrate through the endothelial junctions (**a**). Alternatively, a vascular endothelial cadherin (VE-cadherin) and angiopoietin-1 receptor (TIE2) are cleavage by metalloproteinase-12 (ADAM12), which leads to disruption of endothelial junctions (**b**). Cancer cells moving to blood vessels are also promoted by tumor-associated macrophages (**b**) by secreting epidermal growth factor (EGF). Retraction of endothelial junctions, that facilitate cancer cell transendothelial migration (TEM), might be induced by transforming growth factor β1 (TGFβ1) secreted by cancer cells (**c**). That process can be stimulated by macrophage-secreted tumor necrosis factor 1α (TNF1α) as well. Transcellular intravasation is observed in sites of cancer cell attachment. There complexes of Ca^{2+} –calmodulin induce phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK) causing actomyosin contraction. Finally, that pathway results in creation of transitory pore-like structure enable cancer cell to cross the EC barrier

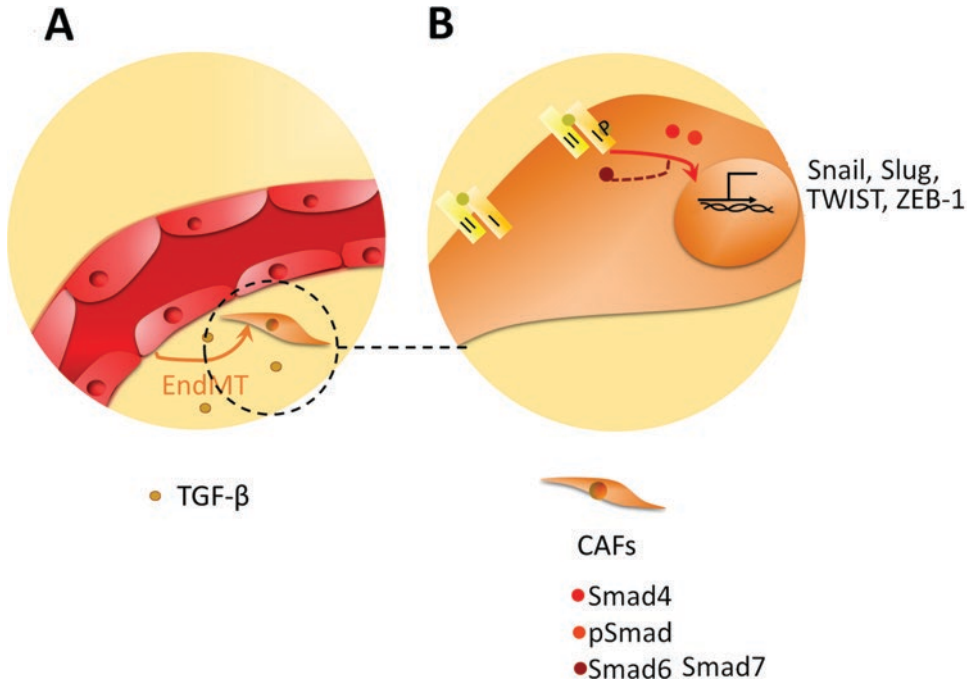

Fig. 6.3 Endothelial-mesenchymal transition. During the tumor progression, the tumor cells secrete TGF-β (**a**), which affects EndMT. TGF-β by stimulation the located in cell membrane TGF receptors (activated after phosphorylation (P) TGFβRI (I) and constitutively active TGFβRII (II)) leads to the activation of Smad proteins, which translocate to the nucleus (**b**). The Smad proteins induce expression of Snail and Slug, TWIST, and ZEB-1 transcription regulators. These transcription modulators cause an increase in the expression of mesenchymal markers and numerous cytoskeletal proteins, leading to the elongation of the endothelial cell and induced its invasive character (as described in the text)

via its phosphorylation [90]. The last of the receptors binds and phosphorylates Smad2/3 which made a complex with Smad4. The created transcription complex moves to the nucleus and triggers the expression of numerous genes which are specific for EndMT [40, 70] such as NOTCH1, TWIST1, and SNAI1/2 [102]. The in vitro observation was confirmed during in vivo analysis on mouse models. The knockdown and knockout of several TGF-β signaling-related genes, such as SMAD2, SMAD3, and TGFBR2, prevented EndMT [28, 115].

TGF-β signaling might be induced indirectly by caveolin-1 (CAV1), Wnt pathway, and endothelin-1 (ET-1). CAV1, located in caveolae, is involved in the internalization of TGF-β receptors [32]. It has been shown that its expression is upregulated during cancer progression. In vivo studies demonstrated that lack of CAV1 induced spontaneous EndMT in mice model. Additionally, TGF-β might accelerate the process [62]. Wnt proteins are involved in EndMT by Smad-dependent TGF-β signaling. They can modulate the phenomenon through canonical (i.e., involving β-catenin) and non-canonical Wnt signaling pathways [4, 63, 107]. Although numerous studies demonstrated that Notch signaling work together with TGF-β pathway [21, 42, 79, 107], it has been shown they act independently in development of Kaposi's sarcoma-associated herpes virus [46]. It has been recently revealed that ET-1, which is an endogenous vasoconstrictor polypeptide, might alone or together with TGF-β cause EndMT in human ECs [25, 113, 114].

Cellular elongation and acquisition of migration ability observed during EndMT correlate with cytoskeleton remodeling. The alterations concern to all types of cellular filaments such as

microfilaments, microtubules, and intermediate filaments. EndMT is characterized by a gain of vimentin expression, which was described as a marker of mesenchymal cells. The regulation of actin cytoskeleton is controlled by proteins belonging to the Rho GTPase family (RhoA, RhoB, Rac-1, cdc42) whose activity is regulated by TGF-β signaling. Activation of small G-proteins causes incorporation of globular actin proteins (G-actin) into filaments of F-actin. This process is critical to forming the stress fibers and results in an increased contraction ability of CAFs [87]. The G-actin pool is released from cytosolic complexes with MRTFs (MRTF-A and MRTF-B) which in "actin-free stage" translocate to the nucleus. MRTFs are the well-described coactivators of serum response factor (SRF) which regulate expression of cytoskeleton regulators and focal adhesion protein such as FAK, vinculin, and α-SMA, necessary for shaping the mesenchymal and contractile nature of CAFs [81]. In our studies, we found that activation of MRTFs are dependent on RhoA and Rac-1/MMP-9 and finally induce ILK and vinculin expression [26]. That axis regulates generation and maturation of focal adhesion, which is characterized by accelerated cell movement, typical for EndMT.

Microtubules, the largest cytoskeleton fibers are involved in the translocation of newly expressed mesenchymal markers, one of which is N-cadherin [68]. It has been proposed that the alteration of β-tubulin subunit expression modulates microtubule dynamics [44]. We revealed that upregulation of tubulins β-3 and β-4 levels, during EndMT, is critical for faster CAFs movement [110, 111].

6.6 Perspective: Endothelium as the Therapeutic Agent in Anticancer Therapy

6.6.1 Antiangiogenesis [AA] Therapies

A variety of signaling molecules such as VEGF-VEGFRs, ephrin-Eph receptors, angiopoietin-Tie, and the Delta-Notch play important roles in angiogenesis. These vascular endothelial growth factors and their receptors regulate both vasculogenesis and pathological angiogenesis. The VEGF family members, i.e., VEGF-A/VEGF-B/VEGF-E and PlGF, regulate angiogenesis and vascular permeability by activating receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice). VEGF-C/VEGF-D and their receptor VEGFR-3 (Flt-4) are mainly observed in lymphangiogenesis. VEGFR-2 is a major signal transducer for neovascularization by the activation of the MAPK signaling pathway. VEGF-A, which demonstrates a variety of functions, including proangiogenic and vascular permeability activity, is the main player. Due to this fact, the VEGF-VEGFR system is an important target for antiangiogenic therapy in cancer progression [94, 104]. Currently, there are four main approaches targeting at cancer angiogenesis tested in clinical trials and approved for clinical practice: (1) neutralizing monoclonal antibody that binds circulating VEGF; (2) recombinant protein (decoy receptor or VEGF-Trap) that binds more than one proangiogenic growth factor; (3) small-molecule tyrosine kinase inhibitors that block tyrosine kinase activity of VEGFRs; and (4) therapeutic monoclonal antibodies targeting VEGFR-2 [72, 78].

One of the first antiangiogenic therapies was a therapy with a humanized monoclonal antibody, neutralizing circulating VEGF-A, i.e., Bevacizumab (Avastin®, Roche/Genentech). The first phase III trial results showed that Bevacizumab combined with chemotherapy in metastatic colorectal cancer (MCRC) improved progression-free survival (PFS) (10.6 vs. 6.2 months) and overall survival (OS) (23 vs. 15.3 months) compared to chemotherapy [54]. Aflibercept (Zaltrap ®, Sanofi Genzyme) is a human recombinant fusion protein that acts as a decoy receptor of VEGF-A, VEGF-B, and PlGF. Aflibercept treatment was approved in MCRC with infusional fluorouracil, leucovorin, and irinotecan [24]. Tyrosine kinase inhibitors (TKIs) are small-molecular-weight drugs that inhibit the kinase activity of different receptors and their downstream signaling. Sorafenib (Nexavar®, Bayer/Onyx) or Sunitinib (Sutent®, Pfizer) target not only at VEGFR but other kinases such as PDGFR and FGFR [78]. Ramucirumab (Cyramza® Eli Lilly) is a human monoclonal anti-

body that inhibits angiogenesis by blocking binding VEGF to the extracellular domain of VEGFR2. It is recommended in combination with FOLFIRI (folinic acid, 5′-fluorouracil and irinotecan) in MCRC patients if the disease progresses after therapy with Bevacizumab, oxaliplatin, and fluoropyrimidine [8].

Direct suppression of tumor angiogenesis and vascular normalization results in suppression of tumor growth. However, after a long-term therapy, tumor cells acquire a resistant phenotype as a result of hypoxia and low nutrition stress. Overall, the survival benefits of antiangiogenic (AA) drugs have not been impressive and surprisingly most cancer patients stop responding or do not respond to the AA therapy at all. What is more, recently it was shown that AA drugs cause a switch to vasoinvasion of tumor cells, leading to increased metastasis and shortened life in mice [39]. The tumor resistance to AA agents can partly be a consequence of non-sprouting mechanisms of vessel recruitment. In intussusceptive microvascular growth, new vessels are generated by creating columns from connective tissue within the lumen of existing vessels. Glomeruloid angiogenesis is characterized by tight nests of vessels that resemble renal glomerulus. In vessel co-option, tumor cells incorporate host vessels in the normal surrounding tissue, and vasculogenic mimicry tumor cells directly from perfused channels bind to the host vasculature. In turn, in the case of looping angiogenesis, contractile myofibroblasts pull host vessels into the cancer tissue [78].

Several phase I and II studies targeting at fibroblast activated protein (FAP) with a humanized monoclonal antibody (Sibrotuzumab) failed to produce clinical benefits in the colon and non-small-cell lung cancer alone or in combination with docetaxel. The latest proposed strategy is based on a specific location of FAP which can be used for precise administration of cytotoxic prodrugs. This strategy is expected to enhance efficacy of the drug delivered to the tumor microenvironment [14].

VEGFR-2 is known as a target for Sunitinib which is a receptor tyrosine kinase inhibitor. ECs transfected with miR-221/miR-222 and treated with Sunitinib showed a reduction in total tube length, and enhancement of cellular proliferation was observed. Sunitinib was not able to abolish the effect of miR-221/222 at pharmacologically relevant concentrations. Such resistance to treatment with Sunitinib may develop when the targeted protein is not accessible for the drug binding. In therapeutic implications, inhibition of miR-221 and miR-222 might improve the patient's survival if administered as an adjuvant therapy in combination with Sunitinib [57].

6.6.2 Inhibition of CAF Formation

Currently, tumor immunomodulation is the main focus of anti-cancer therapies [64]. CAFs, being an important element, regulate cancer invasiveness and characterize by a wide range of cross-talk with other cells located in tumor microenvironments, are a target of anti-cancer therapies. In contrast to preinvasive stages of cancers, the cross-talk processes are mainly observed in invasive cancer stages [64]. That interaction seems to be the main source of chemoresistance. CAFs highly express chemoresistance receptors like retinoic acid receptor β, which improves therapeutic responses of the cells. Cell surface molecules CD10 and GPR77 expressed on CAFs, also contribute to chemoresistance through supporting cancer stemness. Hence, the effectiveness of anticancer therapies in preinvasive stages may not be disturbed but the treatment may additionally intensify tumor growth in invasive stages. Therefore, complexed therapies should be applied.

TGF-βs are the main EndMT inductors contributing to formation of CAFs. Thus, the inhibition of TGF-β pathway seems to be the most promising strategy to decrease the population of CAFs. Theoretically, three levels of inhibition are possessed: (i) ligand inhibition which prevents TGF-β synthesis, (ii) ligand-receptor interaction blocking, and (iii) restriction of signal transduction. Despite the numerous tested inhibitors, studies on their functions mainly focused on modulation of cancer cells. Only one study demonstrated the role of the inhibitor on the cancer niche. TGFβ-activated microenvironment increases the metastasis ability of colon cancer

cell into lungs and liver. Zhang et al. [122] revealed that LY2109761 significantly reduces liver metastases and prolongs survival (by about 25%) in a mouse model. Galunisertib treatment resulted in a blocked formation of subcutaneous tumors by primary colorectal cancer stem cells [19]. Results of these studies demonstrated that STAT3 signaling enhances liver and lung metastasis through TGF-β and IL-11-dependent pathways. The authors prove that targeting at TGF-β signaling can alter cancer cells via cells located in the tumor microenvironment.

Finding effective therapies which would inhibit CAF formation or block their effect on cancer progression appears to be quite difficult.

As described above, CAFs are a heterogeneous group of cells, formed from several sources under the influence of different immunomodulators. Therefore, inhibition of only one pathway is not strong enough to counteract an occurrence of CAFs. Secondly, particular CAF subpopulations demonstrated different functions. It has been recently shown that depending on the location in the cancer niche, CAFs can regulate cancer cells contraction (CAFs located in the close area or within the tumor) or affect the tumor through secreted immunomodulators. Additionally, it has been suggested that different levels of the α-SMA marker, demonstrated by CAFs, depend on the origin of these fibroblasts [70]. Expression of particular markers is another problem that should be considered while searching for anti-CAF therapy. Numerous studies revealed that their expression is dependent on the CAF source. Difficulty is that the presence of particular markers is not specific to CAFs, but it can be observed in other cells of the tumor niche, especially macrophages or lymphocytes [108].

6.7 Conclusion

The endothelium plays a critical role in cancer progression. Inhibition of cancer vascularization, intravasation of cancer cells, and CAF formation are the main reasons for creating effective anticancer therapies and, above all, inhibition of metastasis.

Nevertheless, many strategies limiting the growth of blood vessels proved to be ineffective. The reasons for that should be explained by diversified vasculature of the types of recruited inducing cells or those localized in the tumor area as well as possibilities of their interaction. It should be emphasized that the above-mentioned processes can be induced by tumor cells and tumor niche cells in response to the applied treatment. These elements should be included in the search for new effective therapies that inhibit tumor vascularization. Intravasation of cancer cells could be limited in three ways: by preventing cancer vascularization; by blocking an invasion of tumor cells into the vessel, i.e., by maintaining cell-cell connections; and finally, by inhibiting tumor cells invasiveness. Unfortunately, the last two possibilities are practically not used in anti-cancer therapies. CAFs may serve as the last mechanism that may constitute a source of anti-cancer strategies. It is known that these cells significantly contribute to a development of the tumor, either by induction of proliferation in preinvasive cancer stages or through the acceleration of EMT and metastatic capacity in invasive tumor stages. EndMT inhibition, which accounts for approximately 40% of CAFs, could prevent these adverse effects of CAF formations. However, so far, the role of mechanisms conditioning the transformation and functions of individual CAF subpopulations have not been clearly clarified.

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7 Lymphatic Endothelial Cell Progenitors in the Tumor Microenvironment

Sophia Ran and Lisa Volk-Draper

Abstract

Tumor lymphatics play a key role in cancer progression as they are solely responsible for transporting malignant cells to regional lymph nodes (LNs), a process that precedes and promotes systemic lethal spread. It is broadly accepted that tumor lymphatic sprouting is induced mainly by soluble factors derived from tumor-associated macrophages (TAMs) and malignant cells. However, emerging evidence strongly suggests that a subset of TAMs, myeloid-lymphatic endothelial cell progenitors (M-LECP), also contribute to the expansion of lymphatics through both secretion of paracrine factors and a self-autonomous mode. M-LECP are derived from bone marrow (BM) precursors of the monocyte-macrophage lineage and characterized by unique co-expression of markers identifying lymphatic endothelial cells (LEC), stem cells, M2-type macrophages, and myeloid-derived immunosuppressive cells. This review describes current evidence for the origin of M-LECP in the bone marrow, their recruitment tumors and intratumoral trafficking, similarities to other TAM subsets, and mechanisms promoting tumor lymphatics. We also describe M-LECP integration into preexisting lymphatic vessels and discuss potential mechanisms and significance of this event. We conclude that improved mechanistic understanding of M-LECP functions within the tumor environment may lead to new therapeutic approaches to suppress tumor lymphangiogenesis and metastasis to lymph nodes.

Keywords

Bone marrow · Breast cancer · Endothelial cell lineage development · Hematopoietic stem cell differentiation · Inflammation · Lymphangiogenesis · Lymphatic metastasis · Lymphatic endothelial progenitors · M2-type macrophages · Myeloid-derived pro-vascular progenitors · Myeloid-derived suppressor cells · Tumor macrophages · Toll-like receptor 4 · Tumor microenvironment · Vessel formation

7.1 Introduction

The lymphatic system consisting of lymph nodes (LNs) and the highly organized hierarchal network of lymphatic vessels is unique in the sense

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_7

that it is an integral part of both the body's immune defense and circulatory networks. As part of the immune defense, the lymphatic system is primarily responsible for transporting macrophages and dendritic cells (DC) from the tissues to regional lymph nodes where they present newly harvested antigens to regulatory and effector cells to help mount an adaptive immune response [4]. Lymphatic vessels also play important roles in the leukocyte trafficking and regulation of local immune responses [7, 89, 104]. As part of the circulatory system, lymphatic vessels are responsible for absorbing excessive protein and fluid from the interstitium and returning them to blood circulation [95]. This is particularly important during inflammation that is characterized by elevated vascular permeability [24] and, hence, a significant increase in water and blood proteins in the affected tissues. Specialized lymphatic vessels perform a variety of critical physiological functions in the skin, guts, and other organs [81].

The functions of the normal lymphatic system are beneficial for homeostasis, immune defense, and tissue restoration post-injury. Whereas induction of tumor lymphatics follows the same incentives as physiological lymphangiogenesis, tumor-induced lymphatics play a largely negative role. This is because tumor lymphatics are sole contributors to transporting malignant cells to local lymph nodes, a process that greatly increases systemic metastasis [12, 87]. An additional factor is that in the cancer environment, demands for generation of new vasculature are aggravated by high concentrations and imbalance of endothelium-promoting proteins overexpressed by malignant cells.

The two main factors that induce tumor and inflammatory lymphangiogenesis are vascular endothelial growth factor C (VEGF-C) and a related protein VEGF-D [55]. Both ligands bind the high-affinity tyrosine kinase receptor VEGFR-3 that is primarily expressed in lymphatic endothelial cells (LEC) [68]. VEGFR-3 activation increases proliferation, migration, and morphogenesis of LEC culminating in formation of new sprouts derived from the "mother" vessel. This canonical understanding of lymphatic vessel (LV) formation [27, 72] is now rapidly expanding by the emerging evidence indicating the critical contribution of lymphatic endothelial cell progenitors (LECP) [86, 88].

Although the existence and functional significance of LECP for lymphatic formation were debated in early studies [40, 48], it is now broadly accepted in the field [52, 77, 88]. Addition of exogenous LECP has been shown to increase lymphatic vessel density (LVD) in multiple in vivo models of inflammation [43, 64] and tumors [113], whereas ablation of bone marrow (BM)-derived mononuclear cells inhibits formation of new lymphatics [28]. Myeloid cell-derived LECP (i.e., M-LECP) appear to be the predominant type of lymphatic progenitors that contribute to inflammatory [77] and tumor [88] lymphangiogenesis in both human pathologies [110] and mouse experimental models [113]. Blood-circulating LECP are present at substantially higher levels in cancer patients compared with healthy subjects [9, 85, 113]. As we recently reported, the density of tumor-infiltrating M-LECP in clinical breast cancers significantly correlates with tumor-induced lymphatics and patient lymph node (LN) status [112]. This collective evidence strongly suggests an important role of BM-derived lymphatic progenitors in generation of tumor lymphatics and subsequent metastasis. This review summarizes the current knowledge in the LECP and M-LECP field with particular focus on their recruitment to tumors and interactions with the cells of the tumor microenvironment (TME).

7.1.1 Bone Marrow (BM) Origin of M-LECP

Adult LECP reportedly originate from various sources including the adipose tissue [118], cord blood [107, 110], mesenchymal stem cells [25], and hematopoietic stem cells [53]. However, most studies identified BM-derived immature CD11b-positive myeloid cells as an M-LECP primary source [28, 45, 63, 71, 90]. Supporting the myeloid origin, human blood-circulating mononuclear cells expressing lymphatic markers often co-express CD14, a specific marker of monocytes [19, 60, 110]. BM as the main source of M-LECP

is also indicated by studies that showed reduction of myeloid-lymphatic cells upon depletion of BM cells by gamma irradiation and enhanced lymphangiogenesis upon administration of exogenous BM precursors [90]. Additional support is provided by the studies that showed detection of green fluorescent protein (GFP) in newly formed lymphatic vessels in mice following adoptive transfer of BM cells with constitutive GFP expression [88, 90]. It is also consistent with the known immature status of myeloid-lymphatic hybrid cells indicated by the absence of CD80 [45], a marker of mature macrophages, and high expression of a monocytic progenitor marker Ly6C [113]. Human LECP also express stem/progenitor markers such as CD133 as shown in VEGFR-3$^+$ blood-circulating progenitors in both healthy subjects [19, 94] and cancer patients [9, 110]. Collectively, these reports strongly suggest that M-LECP are derived from BM myeloid progenitors rather than local tissue-differentiated macrophages.

7.1.2 Identification of M-LECP in Clinical Cancers and Experimental Tumor Models

M-LECP circulating in the blood or infiltrating tumors can be identified by combined immunostaining for three types of markers typically segregated to distinct lineages or different stages of maturation:

1. Specific markers of the myeloid lineage (e.g., CD11b in mouse and CD68 in human) indicating their origin
2. Specific markers of lymphatic endothelial lineage (e.g., VEGFR-3, LYVE-1, and podoplanin (PDPN)) indicating the destination of their cell fate
3. Stem/progenitor markers indicating their early differentiation status.

Mouse stem/progenitor markers associated with M-LECP include Sca-1 [63] and Ly6C [111], whereas human lymphatic progenitors were reported to express PU.1 [112], CD133, and CD34 [85, 94]. Co-expression of Ly6C, PU.1, and other stem cell markers in LEC-positive hematopoietic cells suggests that M-LECP are derived from the early precursors of the monocytic lineage because these markers are largely absent in mature myeloid cells [73, 114].

The presence of M-LECP in experimental tumor models has been shown in numerous studies by co-staining for CD11b, a specific marker of monocytes and macrophages, and one or more lymphatic markers. The most consistent lymphatic markers identifying mouse M-LECP are LYVE-1 [51, 96, 123] and podoplanin (PDPN) [63], whereas VEGFR-3 and PROX1 are less reliable due to their low or absent expression. This might be due to differential stages of maturity of tumor-recruited M-LECP. As we previously showed, VEGFR-3 signaling is required only for induction of pro-lymphatic differentiation characterized by upregulated LYVE-1 and PDPN but not for maintaining this lymphatic phenotype [43]. This is in contrast with mature LEC that express VEGFR-3, LYVE-1, and PDPN constitutively. Therefore, it stands to reason that LYVE-1$^+$ and PDPN$^+$ tumor-associated macrophages (TAMs) representing more mature LECP are detected at greater quantities than VEGFR-3$^+$ or PROX1$^+$ M-LECP, owing to the transient expression pattern of these markers during differentiation. Some examples of intratumoral mouse and human M-LECP identified by double staining using myeloid, stem, and lymphatic cell markers are shown in Figs. 7.1 and 7.2.

In human clinical tumors, M-LECP have been similarly identified by co-staining for LEC markers and CD68 that is broadly expressed in most myeloid cells [41], or CD14, a specific monocytic marker [121]. For instance, VEGFR-3-positive cells co-expressing CD14 and CD68 were shown in clinical cervical cancers [97], and LYVE-1$^+$/CD68$^+$ macrophages were detected in human melanoma [33]. We recently showed [112] that 100% of LYVE-1$^+$ and PDPN$^+$ cells infiltrating clinical breast cancers co-expressed classic monocyte-macrophage markers CD14, CD11b, CD18, MD2, MyD88, and Toll-like receptor 4 (TLR4) (Table 7.1). It is important to note that the first four markers are essential components of the TLR4 membrane complex,

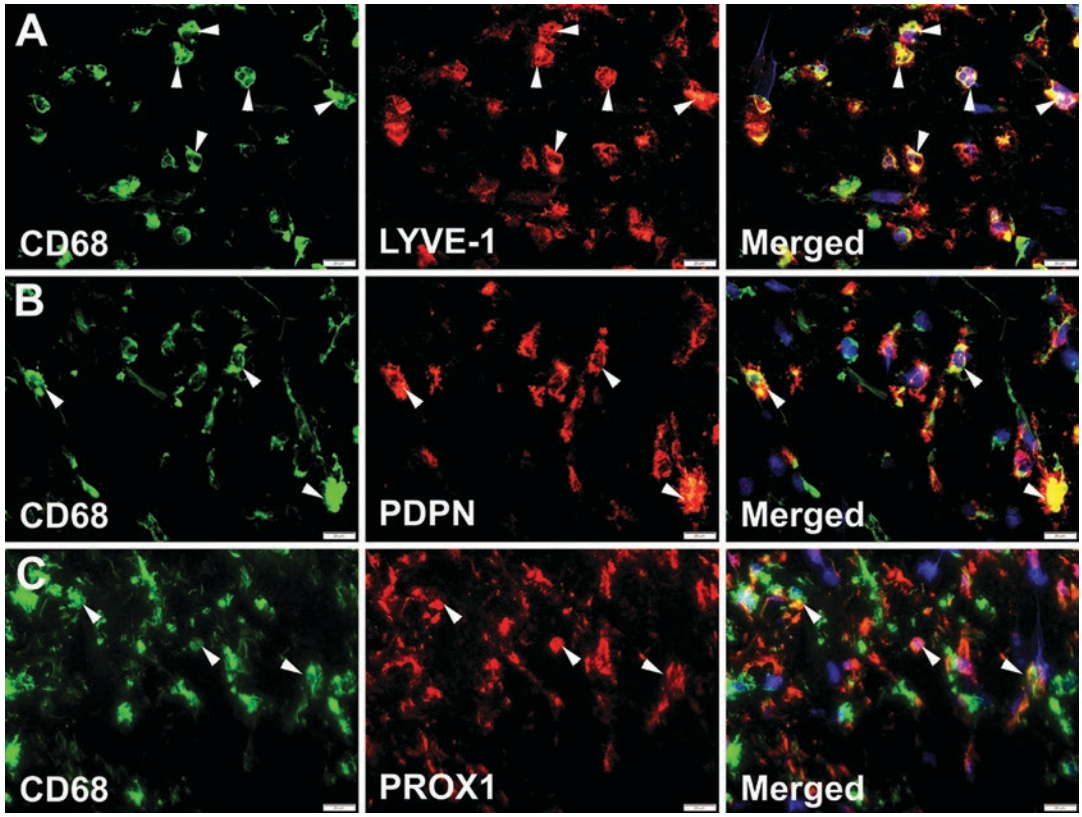

Fig. 7.1 Human clinical breast cancers massively recruit M-LECP. Human BC specimens were co-stained for CD68 (green) and antibodies against markers of lymphatic vessels (red) including (**a**) LYVE-1, (**b**) PDPN, and (**c**) PROX1. Nuclei in merged images were identified by Hoechst stain. White arrowheads indicate cells that co-express CD68 and lymphatic markers. All images were acquired at 600× magnification

whereas the fifth marker (MyD88) is a major intracellular adapter of the activated TLR4. We previously showed that the TLR4 pathway plays a critical role in M-LECP differentiation [43, 113]. Therefore, this profile not only confirms the myeloid-macrophage identity of lymphatic progenitors but also demonstrates a direct link between the TLR4 pathway and lymphatic progenitors recruited to human cancers.

7.1.3 M-LECP Recruitment to Tumors and Their Intratumoral Trafficking

Because M-LECP are hybrid cells with dual myeloid-lymphatic phenotype, they express many chemokine receptors typical of macrophages [113]. It is therefore likely that tumor recruitment of M-LECP is mediated by similar chemoattraction pathways that mobilize other macrophage subsets. For instance, CSF1, one of the most potent monocyte attractants [31, 65], has been shown to recruit LYVE-1$^+$ macrophages in a mouse osteosarcoma model [62]. Interference with CSF1 signaling using a CSF1R inhibitor, PLX3397, reduced TAM infiltration and lymphatic vessel density in a mouse breast cancer model MMTV-PyMT [112]. This suggests that LYVE-1$^+$ macrophages follow the same tumor recruitment pathway as other BM-derived monocytes. A separate study showed that PLX3397 treatment of MMTV-PyMT-bearing mice not only reduced tumor infiltration by BM monocytes but also reduced metastasis [31]. Taken together, these studies suggest a direct link

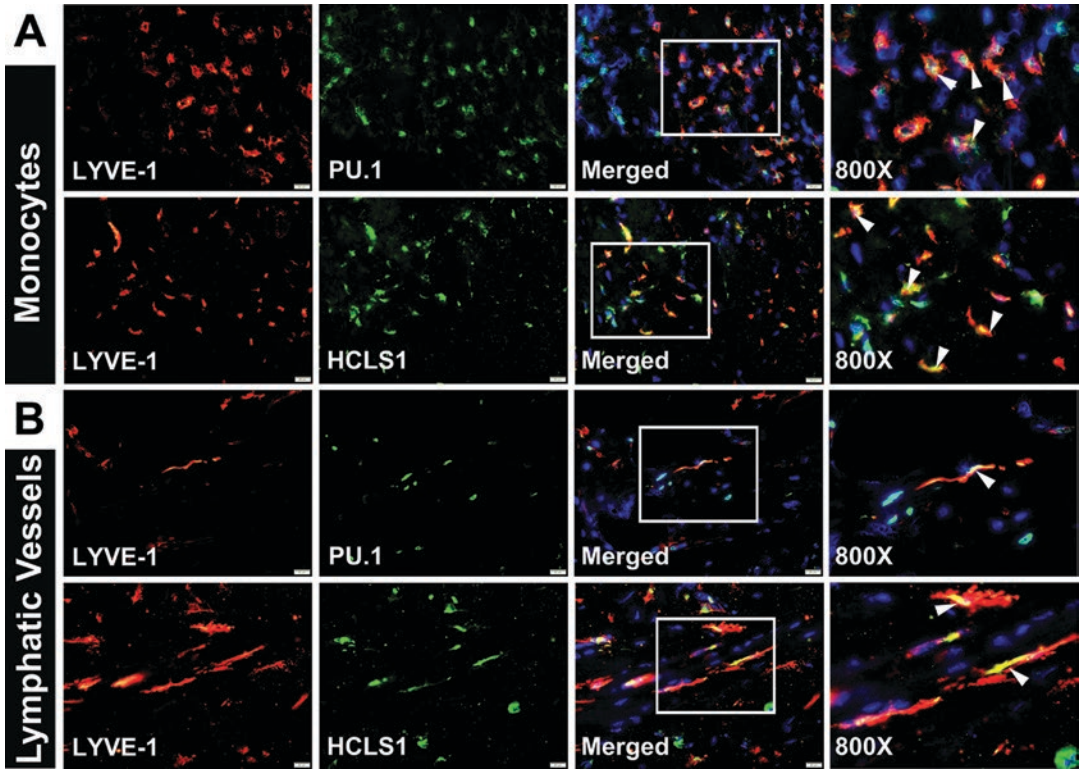

Fig. 7.2 Both tumor M-LECP and lymphatic vessels in clinical breast cancers express stem/progenitor markers. BC specimens were co-stained with anti-LYVE-1, a marker of lymphatic vasculature, and hematopoietic stem markers PU.1 or HCLS1. Both markers were observed in (**a**) LYVE-1$^+$ monocytes and (**b**) tumor lymphatic vasculature. All images were acquired at 400× magnification, with Hoechst stained nuclei present in merged images and 800× magnification panels. White boxes indicate areas highlighted in images taken at 800× magnification. White arrowheads point to cells and vessels expressing both LYVE-1 and stem cell markers

between recruitment of LYVE-1$^+$ macrophages and tumor spread.

Another possible recruiter of M-LECP is VEGF-A, a common tumor-derived factor that promotes both angiogenesis and lymphangiogenesis [116]. VEGF-A plays a major role in the recruitment of BM monocytes via activation of one of its receptors, VEGFR-1 [74]. Consistent with the notion that M-LECP are recruited along with other BM-derived myeloid cells, VEGF-A has been shown to significantly increase the density of lymphatic progenitors in mouse models of human gastric, colorectal, and breast cancers [108]. In line with this report, VEGF-A neutralizing treatment of mice with MDA-MB-231 breast tumors reduced TAM infiltration concomitant with inhibition of lymphangiogenesis [116]. Consistently, treatment of patients with lung, breast, and colorectal cancers using anti-human VEGF-A antibody, bevacizumab, significantly reduced blood-circulating levels of immature myeloid cells [76] that represent a major source of M-LECP [88]. This suggests that VEGF-A targeting might be useful for inhibiting tumor infiltration of M-LECP and subsequent lymphangiogenesis in clinical settings.

Additional candidates for tumor recruitment of M-LECP are CXCL12 (SDF-1), a chemokine shown to recruit LYVE-1$^+$ macrophages to adipose tissue via activation of its receptor CXCR4 [23], and CXCR3, a receptor for chemotactic factors CXCL9, CXCL10, and CXCL11 [91]. The potential for the latter receptor to control M-LECP migration is suggested by similar effects on various immune cells including monocytes [15] and mesenchymal stem cells [42]. Both CXCR3 and CXCR4 have been shown to

Table 7.1 Protein expression profile of LYVE-1$^+$ progenitors in clinical breast cancer

Protein expressed in LYVE-1$^+$ cells	Marker description or alias	Marker lineage expression	% marker positive of total LYVE-1$^+$ cells	Comments
TLR4[a]	Toll-like receptor 4	Myeloid, monocytes, macrophages	100%	TLR4 regulates differentiation of M-LECP [88]
CD11b[a]	CD11b	Myeloid, monocytes, macrophages	100%	CD11b is an essential co-receptor for TLR4 [79] and a marker of myeloid lineages [1]
CD14[a]	CD14	Myeloid, monocytes, macrophages	100%	CD14 is an essential co-receptor for TLR4 [39] and a specific marker of monocytes [121]
MD2[b]	Ly96	Myeloid, monocytes, macrophages	100%	MD2 is an essential co-receptor of TLR4 [13]
MyD88[a]	Myeloid differentiation factor 88	Myeloid, monocytes, macrophages	100%	MyD88 is a key intracellular mediator of the activated TLR4 pathway [22]
CXCR3[b]	CXCR3	Monocytes, macrophages, stem cells	100%	CXCR3 is a chemotactic receptor for stem cells [42], monocytes [15], and other immune cells [67]
STAB1[b]	Stabilin-1	M2-type macrophages LEC	100%	A marker of M2-type macrophages and lymphatic endothelial cells [57, 92]
CD38[a]	CD38	Early progenitors	80%	A specific marker of early BM progenitors [2]
HCLS1[a]	Hematopoietic cell-specific Lyn substrate-1	Early progenitors	50%	A specific marker of early BM progenitors [100]
PU.1[a]	Spi-1-proto-oncogene	Early myeloid progenitors	50%	A key determinant of myelomonocytic differentiation [75]
CD146[b]	CD146	Blood vascular endothelial cells (BEC)	0%	A marker of blood vessels [35] and endothelial progenitors [30]; its absence suggests divergence from BEC lineage
CD3, CD4, CD8[a]	CD3, CD4, CD8	T-cells	0%	Absence of T-cell markers suggests lack of involvement of this lymphoid lineage
CD19[a]	CD19	B-cells	0%	Absence of B-cell markers suggests lack of involvement of this lymphoid lineage
FPR-1[b]	Formyl peptide receptor 1	Mainly neutrophils	0%	A specific marker of neutrophils [82]; the absence suggests divergence from granulocyte lineage
EMA[a]	Cytokeratins	Epithelial cells	0%	Absence of this marker suggests lack of involvement of the epithelial lineages

[a]Data are taken from the reference [112]
[b]Unpublished data

promote lymphangiogenesis [59, 120] and metastasis [59, 122], which is consistent with their potential role in the recruitment of M-LECP. CXCR3 and, to a lesser degree, CXCR4 were detected in all analyzed M-LECP in our study of clinical breast cancers (Table 7.1). However, the direct chemotactic role of either CXCR3 or CXCR4 in tumor M-LECP mobilization has not been determined.

Upon arrival to tumors, M-LECP tend to accumulate near tumor lymphatic vessels [26], implying the existence of an intratumoral chemotactic

gradient generated by LEC. This is not surprising because macrophages and DC commonly use lymphatic vessels to exit inflamed tissues on their journey to regional LNs [6, 17]. M-LECP retain the myeloid phenotype along with expression of lymphatic markers and therefore may use LV-generated chemotactic gradients of CCL19/CCL21 known to attract CCR7$^+$ monocytes and dendritic cells (DC) [93, 105]. Monocyte-attracting chemokines CCL2, CCL3, and CCL5 might also be involved in M-LECP recruitment to tumors in general and to lymphatic vessels, specifically. This is supported by detection of the corresponding receptors of CCL2, CCL3, and CCL5 in M-LECP differentiated in vitro [113]. These cytokines have also been shown to attract blood vascular endothelial progenitors to intratumoral vessels [102], suggesting a similar role in recruitment of LECP. However, their pro-migratory functions in the context of lymphatic progenitors and vasculature have not been directly analyzed.

7.1.4 Relationships Between M-LECP and M2-TAMs

Tumor-associated macrophages (TAMs) are customarily divided into M1 (immunostimulatory) and M2 (immunosuppressive) types with the latter dominating the TME [99]. Some consider this an oversimplified categorization since many TAMs express both M1 and M2 markers [21, 66, 106] and display functional behavior associated with both types. However, it has been widely confirmed that TAMs express various scavenger receptors such as CD163, CD204, and CD206 that are regarded as specific M2-type markers. Scavenger receptors are a heterogeneous class of proteins with broad ligand specificity whose main function is to remove foreign elements from the inflamed or wounded tissue. Such proteins are highly upregulated in the type of macrophages responsible for cleansing and remodeling an injured site. Not surprisingly, accumulation of toxic material in the pathological TME attracts and retains macrophages expressing scavenger receptors. In relation to M-LECP, many TAMs expressing scavenger receptors also express the lymphatic marker LYVE-1 [36, 96]. TAMs with dual expression of M2 and LEC markers were identified in human clinical melanoma and a mouse B16 melanoma model [33]. TAMs expressing CD206 and another LEC marker, VEGFR-3, were found in syngeneic 4T1 breast tumors [36] as well as in other tumor models [96, 123]. We recently demonstrated in clinical breast cancers that a large fraction of LYVE-1$^+$ TAMs co-express CD163 and CD204 [112]. The overlapping expression of scavenger receptors in TAMs and tumor M-LECP not only confirms the myeloid-macrophage identity of lymphatic progenitors but also suggests a common immunosuppressive nature of both cell types.

While co-localization of LEC markers in M2-TAMs is fairly well established, the underlying reason remains obscure. However, the new understanding that co-signature of M2 macrophages and LEC markers identifies these cells as M-LECP supports a different perspective. As mentioned above, TAM gene expression suggests that their main function is not necessarily to stimulate or inhibit the immune system (they do a little bit of both) but to restore homeostasis disturbed by the TME. A similar macrophage type is found at the resolution phase of wound healing geared toward restoration of the tissue's function after eliminating pathogens and re-creating lost structural components [69]. In such capacity, the M2-macrophages must contain a subset that restores blood vasculature for the obvious reason that no tissue expansion or remodeling can occur in the absence of adequate oxygen and nutrient supply. Angiogenesis is customarily followed by lymphangiogenesis to coordinate fluid and protein balance between the two circulatory systems. Therefore, it stands to reason that M2-type macrophages, the builders of the new site, would contain a subset of pro-vascular cells designated to regenerate both blood and lymphatic vessels. Indeed, TAMs have been repeatedly linked to tumor angiogenesis [20, 70]. Analogously, M2-TAMs expressing LEC markers (i.e., M-LECP) represent a subset of pro-vascular myeloid cells with a specific mission to create new lymphatics.

7.1.5 Relationships Between M-LECP and Myeloid-Derived Suppressive Cells (MDSC)

MDSC are defined as cells that express myeloid progenitor markers and have abilities to suppress functions of T-cells, B-cells, and NK cells [11]. In mouse models, MDSC are identified by $CD11b^+/Ly6C^{low}/Ly6G^+$ (defined as granulocytic PMN-MDSC), $CD11b^+/Ly6C^{high}/Ly6G^-$ (defined as monocytic M-MDSC), or $Gr\text{-}1^+/CD11b^+$ cells representing a mixed type [11]. Human markers for MDSC include $CD14^-/CD11b^+/CD15^+$ (PMN-MDSC) and $CD14^+/CD11b^+/HLA\text{-}DR^{low}$ (M-MDSC) [11]. In both species, MDSC are regarded as BM-derived immature myeloid cells accumulating in tumors due to high turnover of the existing TAMs [103].

Despite their significance, the exact definition of the MDSC phenotype is still evolving due, in part, to selected study methodology. For instance, many studies did not measure presumed MDSC immunosuppressive activity but rather identified tumor MDSC based solely on the surface markers shared with other myeloid subtypes. Additional confusion is caused by extensive use of RB6-8C5 antibody that recognizes the granulocyte differentiation 1 (Gr-1) epitope shared by two isoforms of Ly6 protein, Ly6G and Ly6C [38, 56]. Although Ly6G and Ly6C are co-expressed in early BM precursors, they are later aligned with either a granulocytic or monocytic lineage but not both [49]. The broad use of RB6-8C5 antibody that binds to the mixed Ly6G/Ly6C epitope adds another layer of uncertainty over specific markers that define MDSC.

With that being said, a number of studies did detect a significant overlap between M-LECP markers and those ascribed to MDSC. For instance, VEGFR-3 was detected in MDSC in lymphoid organs and TAMs infiltrating 4T1 tumors [36]. SAR131675, a specific inhibitor of VEGFR-3, was shown to suppress proliferation of TAMs in vitro and reduce their tumor density in vivo [18]. Analysis of clinical breast cancers showed that $TIE\text{-}2^+$ macrophages expressing LEC markers LYVE-1, VEGFR-3, PDPN, and PROX1 exhibited not only pro-lymphangiogenic but also immunosuppressive activity [10]. These cells also co-expressed a monocytic marker CD14 considered as one of defining components of the MDSC signature. PDPN-positive myeloid cells in a mouse glioma model were also shown to possess immunosuppressive activity, and deletion of PDPN from these myeloid cells increased tumor influx of $CD8^+$ cytotoxic T-cells [34]. This evidence collectively suggests that M-LECP, like many other tumor-infiltrating immune cells, suppress the anti-tumor activities of the host.

The potential ability of M-LECP to suppress immune responses might be important for their main function to induce new vasculature. Tumor vascular formation requires complex spatiotemporal coordination for differentiation and recruitment of endothelial and perivascular progenitors as well as intricate interactions with matrix and other cells in the TME. These complex processes might be prohibited in an environment generated by ongoing cytotoxic activities of immune cells, which likely exert bystander effects. It is possible that M-LECP and other pro-vascular progenitors have to be immunosuppressive to execute their functions in order to avoid structural disruption of newly created fragile vessels. Albeit currently speculative, this hypothesis is supported by documented immunosuppression of other sites associated with generation of new vessels such as late stages of wound healing and pregnancy [99].

7.1.6 Interactions of M-LECP with Tumor-Associated Lymphatic Endothelium

One cell type that LECP clearly interact with in the tumor environment is LEC lining preexisting lymphatic vessels. This conclusion is based on two main lines of evidence. First, tumor-infiltrating M-LECP are often found in proximity or close association with preexisting lymphatic vessels [90, 123]. Second, they structurally integrate specifically into lymphatic vessels even if blood vessels are present in the same field [113, 123]. It is also significant that $LYVE\text{-}1^+$ progenitors integrate only into tumor-associated vessels

but not those in nearby nonneoplastic tissues [10]. This suggests coordinated expression of complementary receptors on M-LECP and activated or inflamed lymphatic vessels that control their specific interaction.

Vascular integration of lymphatic progenitors has been tracked and quantified using various approaches. One approach is detection of exogenously introduced markers such as GFP [113, 123] or a fluorescent dye Dil [63] combined with immunostaining for lymphatic-specific (e.g., LYVE-1) and myeloid-macrophage markers such as CD11b and F4/80. An alternative method employed chimera mice reconstituted with the BM from GFP-expressing mice [90, 109, 113] which allows cell fate and lineage tracking of BM-derived cells. Detection of "green" lymphatic vessels that co-express LYVE-1 indicates insertion of the GFP mRNA or protein into new sprouts, which can occur only through physical interaction with GFP-positive BM-derived cells. This event was shown in multiple experimental models including fibrosarcoma [90], Rip1Tag2 insulinoma [123], melanoma [63], MMTV-PyMT breast [113], and TRAMPC-1 prostate [123] cancers. LYVE-1$^+$ cells derived from transplanted GFP$^+$ BM-derived hematopoietic stem cells were identified in intestinal tumors spontaneously developed in Apc (Min/+) mice [53]. BM-derived LYVE-1$^+$ cells co-expressing a stem cell marker CD34 and a LEC marker VEGFR-3 were shown to integrate into peritumoral lymphatic vessels of mouse T241 fibrosarcoma [90]. CD11b$^+$/PDPN$^+$ tumor macrophages were detected in melanoma-associated lymphatic vessels [96]. In line with these reports, we found widespread lymphatic integration of adoptively transferred GFP$^+$ M-LECP differentiated in vitro in a variety of syngeneic breast tumors EMT6 and MMTV-PyMT and xenografts of human breast carcinoma lines MDA-MB-231 and ZR-75 [112, 113]. Integration of LECP and M-LECP into tumor lymphatics in human cancers was shown by demonstrating highly expressed myeloid markers CD14 and CD68 [10, 112]. By contrast, lymphatic vessels in corresponding normal organs express low-level or no myeloid markers [112].

An example of complete M-LECP integration into tumor-associated lymphatic vessels in transgenic mouse MMTV-PyMT model is shown in Fig. 7.3. Confocal analysis showed that LYVE-1 and a macrophage marker F4/80 were co-expressed in the entire thickness of the vessel (Fig. 7.3, b1–b5 images). The same images show co-expression of lymphatic junctional protein VE-cadherin dispersed along the analyzed vessel (Fig. 7.3b). Co-expression of all three markers in the same vascular structure strongly favors coalescence of M-LECP with preexisting LEC rather than insertion of individual progenitors into the vascular wall. We detected in average 50% and up to 90% of tumor lymphatic vessels with myeloid-macrophage markers in both syngeneic and xenograft breast cancer models [112, 113]. Independent studies showed integration in ~60% of lymphatic vessels in LS174T colorectal and SK-BR-2 breast tumors [108]. Similar approaches detected LECP integration into lymphatic vessels in multiple inflammatory models [71, 96] as well as human tissues undergoing inflammatory lymphangiogenesis [60].

These observations are highly reminiscent of integration of blood vascular endothelial progenitors into tumor blood vessels [44] indicating that both blood vascular and lymphatic progenitors might follow the same process during inflammatory or tumor vascular formation. Further support for this conclusion is shown in studies with patients who received gender-mismatched BM transfusion years before tumor development [80]. Intriguingly, analysis of blood vessels in their cancers detected chromosomes from the opposite sex identified by in situ hybridization using specific probes to X and Y chromosomes [80]. Detection of the entire chromosome in the nuclei of tumor endothelial cells (EC) strongly suggests transfer of the whole cellular content of progenitors to existing EC rather than lineage infidelity, transcriptional aberration, or random upregulation of an isolated marker.

Another line of evidence that supports the donation of the entire progenitors' contents is expression of protein tags experimentally introduced in LECP. We showed in both inflammatory [43] and tumor mouse models [113], as well as in

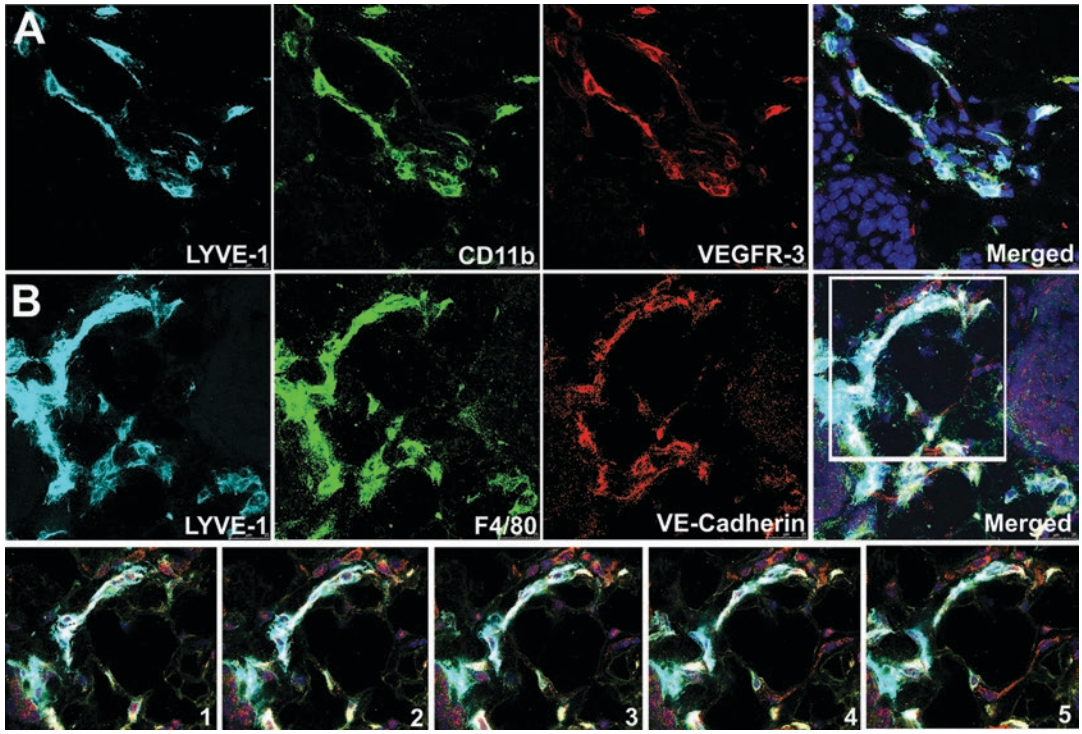

Fig. 7.3 Confocal microscopy analysis shows evidence for M-LECP integration into tumor lymphatic vessels. MMTV-PyMT tumors were triple-stained for LYVE-1 and (**a**) CD11b and VEGFR-3 or (**b**) F4/80 and VE-cadherin. The region highlighted by a white box in **b** indicates the area analyzed by confocal Z-stack represented below in panels 1–5. Each image was captured 2 μm apart. All images were acquired at 1000× magnification

human breast cancers [112], that endogenous myeloid markers and ectopic GFP are dispersed throughout lymphatic vessels after integration of M-LECP. An independent study using a pancreatic RT2 tumor model showed a similar pattern of GFP expression in tumor lymphatic vessels in mice that received a transfer of BM-derived GFP^+ cells [123]. Using confocal microscopy and Z-stack analyses, the authors of this study distinguished among GFP^+ cells closely associated with lymphatic vessels, GFP^+ macrophages transmigrating through the vascular wall, and those truly integrated into the endothelial layer [123]. While all three events have been identified in expanding vasculature, only full integration of lymphatic progenitors into vessels can account for the broad GFP expression pattern in recipient lymphatic vessels [123] and longevity (>1 year) of GFP expression in these structures [53]. Taken together with the evidence described above, this suggests that pro-vascular progenitors might promote sprouting by transferring their cellular contents to the existing endothelium. Currently, however, the mechanisms of vascular integration of progenitors as well as the physiological impetus driving this process remain unknown.

7.1.7 Role of M-LECP in Generation of New Tumor Lymphatic Vessels

Although many aspects of M-LECP-mediated lymphangiogenesis are still poorly understood, three main mechanisms have been proposed in current literature. The most widely accepted concept suggests that myeloid-lymphatic cells promote lymphatic formation by virtue of overexpression of lymphangiogenic factors VEGF-A [108] and VEGF-C [32, 58, 61]. These factors

stimulate, respectively, VEGFR-2 and VEGFR-3 expressed on LEC, and therefore their binding to these receptors is expected to induce the formation of new vasculature [50, 68]. This concept is supported by multiple lines of evidence from both experimental models and clinical studies. For instance, tumor M2-type macrophages [115, 117, 119] and myeloid cells with LEC markers [97] were shown to express much higher levels of lymphangiogenic factors than CD11b-negative cells [117]. Moreover, tumor expression of VEGF-A and VEGF-C is known to correlate with tumor LVD and lymphatic metastasis [8, 78, 98]. This mechanism is also supported by studies demonstrating suppression of tumor lymphangiogenesis by anti-VEGF-A antibody [116] or agents targeting the VEGFR-3 pathway [14, 46, 47, 84]. Suppression of tumor lymphangiogenesis and lymphatic metastasis by global elimination of macrophages also favors this concept [117].

While this evidence is generally consistent with the important role of TAM-produced paracrine factors in vascular formation, this mechanism does not effectively explain several findings, particularly those emerging in the M-LECP field. First, the majority of studies that supported a paracrine effect of VEGF-C did not compare the total amount of VEGF-C produced by TAMs with the amount derived from tumor cells. A single study that did compare the levels of VEGF-C transcripts showed a substantially higher expression in malignant cells compared with macrophages from the same tumor [123]. As shown in this study, for each 100 molecules of VEGF-C transcript expressed by tumor cells, macrophages produced only one to two molecules [123]. We recently confirmed this observation in a human breast cancer xenograft model, MDA-MB-231, by comparing the exact number of mouse and human VEGF-C transcript copies in the same tumor samples. We found that for each molecule of mouse VEGF-C produced by the entire tumor stroma, nearly 1000 transcript copies were produced by human malignant cells [112]. Based on the combined evidence from these two studies, it appears that the minuscule contribution of stroma including TAMs is unlikely to be significant for induction of new lymphatic vessels.

Another argument for the TAM pro-lymphangiogenic role mediated by paracrine factors is based on studies demonstrating inhibition of tumor lymphatics by anti-VEGF-C or anti-VEGFR-3 agents [47, 117]. However, the problem with this argument is that systemic inhibition of VEGFR-3 does not distinguish between local effects inhibiting VEGFR-3 on sprouting vessels and suppression of M-LECP generation in the BM that heavily relies on this pathway [43, 88]. Targeting macrophages in general also does not provide a clear mechanism since such treatment does not discriminate between elimination of soluble factors produced by M-LECP and alternative mechanisms relying on cell-cell interactions. Additional problem to explain the M-LECP role in lymphangiogenesis based only on production of soluble factors is the acquisition of the lymphatic phenotype by differentiated M-LECP [43, 113]. Arguably, VEGF-C transcription that can be induced in fibroblasts, epithelial cells, and other cell types requires no coincident expression of LEC-specific proteins in the producing cells. It is therefore unclear why M-LECP should express LYVE-1 and many other LEC markers if their sole function is to produce VEGF-C. Lastly, this mechanism does not address integration of M-LECP into preexisting LEC, an event that defies a logical explanation if the induction of lymphatics depends only on the paracrine support. This collective evidence argues that a cell-autonomous role of M-LECP might be more important for induction of lymphatic sprouting than their contribution to lymphangiogenic factors, particularly in the context of cancers secreting voluminous amounts of such proteins.

Another suggested mechanism of M-LECP-dependent lymphatic expansion is lymphovasculogenesis, a process similar to generation of primitive lymphatic vasculature during embryonic development. Embryonic vascular formation is fundamentally different from that in the adults by virtue of the absence of preexisting vessels. Vasculogenesis is common during embryogenesis but extremely rare in adulthood. However, two independent studies in cornea injury models showed de novo lymphatic vessels arisen within the avascular limbus stroma at a considerable

distance from preexisting lymphatic vessels [71, 110]. Moreover, the new vessels expressed GFP that could be derived only from GFP$^+$ BM cells transplanted prior to injury [71]. The same study showed that isolated BM-derived CD11b$^+$ cells created LYVE-1$^+$/PDPN$^+$ tubes in vitro [71], demonstrating their ability to replicate lympho-vascular morphology. Similar but rare instances of lympho-vasculogenesis were also observed in a model of peritonitis induced by a TLR4 ligand, LPS [43], and in MDA-MB-231 tumors activated by another TLR4 ligand, a chemotherapeutic drug paclitaxel [111]. The latter observation is potentially significant from a clinical perspective because paclitaxel was able to induce vessels in the center of the tumor normally devoid of lymphatics [111]. Intratumoral lymphatics are highly efficient in mediating metastasis due to proximity to tumor cells [5]. The enhanced LN metastatic burden was, indeed, demonstrated in paclitaxel-treated tumor-bearing mice [111]. Whether lympho-vasculogenesis commonly occurs in clinical cancers is currently unknown.

The third proposed mechanism for M-LECP induction of tumor lymphangiogenesis involves integration of M-LECP into preexisting lymphatic vessels observed during both inflammatory [64] and tumor lymphatic formation [90, 108, 113, 123]. This event was previously described as "incorporation" [53, 90, 109], "integration" [16, 63, 123], or "insertion" [10] of myeloid-lymphatic cells into tumor vasculature. However, a more accurate description might be "fusion." This is because histological and immunohistochemical analyses of tumors in vivo show a complete overlap between myeloid and lymphatic markers in vessels rather than insertion of individual myeloid cells between two adjacent LEC. Several additional lines of evidence also support the theory of M-LECP fusion with LEC. Confocal microscopy analyses showed that myeloid markers derived from M-LECP are detected throughout the length and depth of the lymphatic vascular structures and are not restricted to "inserted" myeloid cells (Fig. 7.3). Chimera mice reconstituted with GFP$^+$ BM generated "green" LV in which GFP was evenly distributed through the entire thickness of the vessels identified by LYVE-1 and VE-cadherin markers [112]. Independent studies showed coalescence of lymphatic progenitors with LEC during inflammatory lymphangiogenesis by detecting Y chromosome in lymphatic vessels in female patients undergoing rejection of gender-mismatched kidney transplants [60]. This is reminiscent of detection of XX and Y chromosomes in the nuclei of tumor (but not normal) blood vascular endothelial cells (BEC) in patients who received gender-mismatched BM transplants years before tumor development [44]. Clearly, the long-term presence of one or more chromosomes in remodeled vasculature indicates not just cell-cell interaction but donation of the entire genomic material, which is difficult to explain by any other mechanism but fusion. In support of this concept, we recently demonstrated that conditions mimicking TME promote fusion of GFP$^+$ mouse macrophage line co-cultured with red fluorescent protein (RFP)-tagged LEC [112]. Fusion was detected by both color overlap (i.e., presence of yellow cells) and shared nuclei [112]. An example of fusion of LEC and inflamed macrophages induced by TME-mimicking conditions in vitro is shown in Fig. 7.4. This assay also detected a substantially increased nuclear multiplication in the fused cells, suggesting that transfer of the M-LECP genomic material to LEC might be necessary for cell division, a key prerequisite for generation of new sprouts.

It should be noted that stem and progenitor cells routinely use fusion for direct transfer of biological material to cells requiring recovery or functional reprogramming [3]. This is particularly noted under injury [29], tumor [83], and inflammatory conditions [54] reminiscent of TME. Fusion and other means of transferring cellular contents are the common mechanisms of stem/progenitor cells recruited to damaged and injured sites that have been programmed to restore the lost components of these tissues [37, 101]. Conceptually, tumor M-LECP are similar to other progenitors attempting to restore functions of the wounded organs. Fusion used by

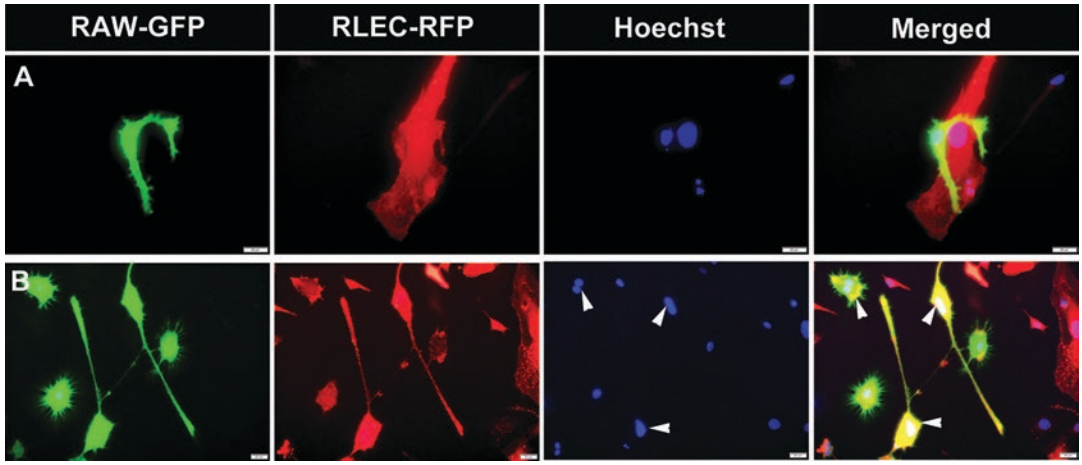

Fig. 7.4 Fusion is a possible mechanism of M-LECP integration into tumor lymphatics. Rat lymphatic endothelial cells expressing RFP (RLEC-RFP) and macrophage cell line RAW264.7 expressing GFP (RAW-GFP) were co-cultured for 4–6 days in serum-free medium containing 3 nM of LPS. (**a**) RAW-GFP migrated to RLEC-RFP displayed intimate cell-cell interactions. (**b**) After 24–48 hours, many cells underwent fusion indicated by yellow color and multi-nucleation highlighted by white arrowheads. Homogenous color throughout fused cells indicates complete donation of the RAW-GFP cell contains to RFP-tagged lymphatic endothelial cells. All images were acquired at 600× magnification

other BM progenitors is the most effective way to provide injured cells in peripheral organs with the survival factors, promitotic signals, and nuclear transcription factors needed to direct structural expansion and to enforce reprogramming. Fusion asserts the breadth, the speed, and the exquisite specificity of delivered factors to the target cells. Such assertion cannot be matched by diffusion of paracrine soluble factors limited in vivo only to a few hundred microns by anatomic barriers. This is particularly relevant to generation of new adult vasculature known for resistance to endothelial cell division under normal circumstances. It is well established that major expansion of either blood or lymphatic vasculature during adulthood is strictly reserved to drastic and unresolved changes in homeostasis such as chronic inflammatory diseases and cancer. It is therefore tempting to suggest that while soluble paracrine TAM-derived factors can aid in new vessel formation, the key mechanism forcing the adult endothelium to undergo highly complex changes required for sprouting must be induced by more drastic cell-transforming mechanism such as fusion. Only fusion can directly deliver genome-remodeling regulatory proteins imposing a fundamentally new behavior on the needed cells. If this theory is proven to be correct in future studies, this might explain how a relatively small number of BM progenitors can produce an extensive network of new vessels.

In summary, currently proposed mechanisms of progenitor-mediated lymphangiogenesis include the following:

1. Production of soluble pro-lymphatic factors directly acting on existing endothelium
2. Embryonic-like lympho-vasculogenesis that does not require preexisting vessels
3. Full donation of the progenitors' contents to LEC mediated by fusion or other means of protein and gene transfer

The latter is suggested to enable the existing LEC to undergo complex processes required for sprouting that are typically prohibited under normal or transient inflammatory conditions to prevent promiscuous vessel formation. A better understanding of the proportional contribution of these mechanisms to, and their collective impact on, the formation of tumor lymphatics is likely to emerge in future studies.

7.2 Future Directions

The M-LECP field is now entering an exciting new phase. In the past decade, inflammation-dependent induction of M-LECP in humans and mice was firmly established. Myeloid-lymphatic progenitors have been shown to mobilize from the bone marrow to sites of inflammation where they significantly contribute to structural expansion and function of new lymphatics, in part, by integration into preexisting vasculature. In the absence of preexisting lymphatics, M-LECP undergo an embryonic-like lympho-vasculogenesis. Throughout these processes, M-LECP retain their myeloid and stem-like identities while adding LEC features without becoming mature endothelial cells. Five outstanding questions that need to be addressed in future studies are as follows:

1. What are the differentiation mechanisms in the BM diverting the myeloid-macrophage precursors toward acquisition of the lymphatic phenotype?
2. Which chemokines are responsible for M-LECP mobilization to the blood, to tumor recruitment, and specifically toward tumor lymphatic vessels?
3. What mechanisms regulate de novo formation of lymphatics via adult lympho-vasculogenesis?
4. What mechanisms control M-LECP integration into the vasculature? What is the nature of this event? What happens after integration?
5. What are the differences and similarities between LECP generated in the BM and those produced by peripheral tissues?

Regarding the first question, the current evidence suggests that M-LECP differentiation requires continuous presence of inflammatory cytokines that promote generation of the macrophage lineage (e.g., CSF1) as well as potent immunomodulators such as TLR4 ligands. However, identification of the specific transcription factors that control myeloid-lymphatic transition still awaits future studies. With regard to the second question, the screening of individual chemokines needs to be conducted to determine whether M-LECP take advantage of classic trafficking pathways of inflammatory monocytes or express their own receptors to direct migration to inflamed tissues. Analysis of the third question will require side-by-side comparison of specific transcription factors and cellular events as well as interaction with the cells in the local environment during embryonic and adult lymphatic formation.

The fourth question is arguably the most intriguing of all because of the paucity of current evidence illuminating the mechanisms of vascular integration of either blood or lymphatic progenitors and the lack of any information regarding the molecular consequences of this event. The physiological impetus for M-LECP to undergo such a process also remains undefined. The only direct clue to this question is the recent evidence suggesting that integration indicated by co-localization of myeloid and lymphatic markers in new vessels might reflect fusion of M-LECP with preexisting LEC [112]. Future studies will need to validate this hypothesis, and if confirmed, tease out specific steps and molecular regulation of this process.

Lastly, future research will need to compare the molecular profiles and mechanisms of differentiation of LECP that originate from other sources than hematopoietic stem cells or myeloid precursors. Such studies should provide critical information for understanding the diversity of M-LECP population and the role of local tissue sources for lymphatic regeneration and remodeling.

In summary, future studies of M-LECP-dependent promotion of lymphatics are expected not only to clarify the mechanisms of tumor lymphangiogenesis and associated metastasis but also to illuminate the consequences of chronic inflammation associated with many human disorders. Such studies should also advance the understanding of the fundamental mechanisms of tissue regeneration during adulthood.

Acknowledgments The authors are grateful to Susan Ryherd for critical review and editing. This manuscript was supported by a grant # R01CA199649 awarded to Sophia Ran by the National Institutes of Health and a Team Science Grant from Simmons Cancer Institute funded by proceeds of the Denim and Diamonds charity event.

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Oligodendrocyte Progenitor Cells in the Tumor Microenvironment

8

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Abstract

Glioblastoma (GBM) develops from adult brain white matter and is the most common and lethal primary brain tumor, characterized by rapid growth and invasion. GBM tumors frequently spread into the contralateral hemisphere, including in the beginning of tumor development. However, after complete resection of the tumor mass and chemo-radiotherapy, GBM commonly recurs around the tumor removal site, suggesting that the microenvironment at the tumor border provides therapeutic resistance to GBM cells. To improve patient prognosis, understanding the microenvironment at the tumor border is critical. Several microRNAs (miRNAs) show higher expression at the tumor border, with the top three involved in oligodendrocyte differentiation. Oligodendrocyte progenitor cells (OPCs) may induce stemness and chemo-radioresistance in GBM cells, providing a supportive function to promote GBM. This review describes important features of OPCs and insights into the "border niche," a unique microenvironment that allows GBM cells to survive and recur at the tumor border.

Keywords

Border niche · Glioma-associated oligodendrocyte · Oligodendrocyte progenitor cell · Oligodendrocyte · Microenvironment · Glioblastoma · Recurrence · Neuron · Microglia · Macrophage · microRNA · Stemness · Chemo-radioresistance · Invasion · Niche

8.1 Introduction

The major cell types in the brain are neurons, glia such as astrocytes, oligodendrocytes derived from the neuroepithelium, and microglia derived from erythromyeloid cells in the yolk sac during the early developmental stage [32, 76]. Glioblastoma (GBM) is the most common primary brain tumor and shares characteristics with glial cells. Despite standard treatment using safe maximal resection and chemo-radiotherapy, GBM generally regrows and/or recurs. The mean 5-year survival rate of GBM patients is less than 10% [62, 74], which has not significantly improved in the past several decades.

The resulting tumor mass is easily detected using gadolinium-enhanced T1-weighted images

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5_8

(Gd-T1WI) in magnetic resonance imaging (MRI). GBM cells invade white matter and migrate into the contralateral hemisphere through the corpus callosum, even in the early stages of tumor progression [86]. Enhanced tumor lesions are surrounded by edema, where invading GBM cells are detected pathologically. In cases in which enhanced tumor lesions are completely removed by surgical operation and chemo-radiotherapy, GBMs typically recur in the white matter around the tumor removal cavity but are rare in areas distant from the primary lesion [9, 26, 66]. This suggests that glioma stem cells (GSCs) [71] which are responsible for recurrence survive in the tissue just outside of the enhanced lesion [26–28]. Biological characterization of this border area between the brain and tumor mass is essential for inhibiting recurrence and removing GSCs, which may improve the prognosis of patients with GBM. Moreover, although GBM invades the white matter, it does not grow toward the empty cavity after tumor resection. These results suggest that the interaction between GBM cells and non-GBM cells is crucial for tumor invasion and regrowth. Unique microenvironments for GSC niches inside the tumor mass have previously been discussed, but studies investigating the outside of the tumor mass are rare [19, 26, 27, 34, 47, 64, 67, 68]. GBM cells and non-GBM cells, including immune cells, neural cells, and brain vascular cells, along with the extracellular matrix, form the GSC niche at the tumor border [26, 28, 64, 70]. Accumulation of oligodendrocyte progenitor cells (OPCs) and microglia/macrophage at the tumor border contributes to the unique GBM microenvironment, promoting stem like characteristics and chemo-radioresistance [26]. The relationship between GBM and microglia/macrophages has been reported previously [2, 26, 48, 65]. This review focuses on the interesting characteristics of OPCs and their interactions with GBM [26, 28], as well as the novel concept of a "border niche" composed of accumulating oligodendrocyte lineage cells (OLCs) named glioma-associated oligodendrocytes (GAOs).

8.1.1 Cells Residing in the Brain Parenchyma

The central nervous system (CNS) is composed of neurons, glia (astrocytes and oligodendrocytes), and microglia. Neurons, astrocytes, and oligodendrocytes originate from neuroepithelial cells; in contrast, microglia are derived from erythromyeloid progenitors in the yolk sac and migrate into the CNS early during development [23, 32, 76] (Fig. 8.1a). Recently, it had been reported that the human brain contains a glia to neuron ratio of less than 1:1, and the total number of glia is less than 100 billion [83]. Roughly, the glial subtypes in human brains are 20% astrocytes, 3–10% OPCs, 25% oligodendrocytes, and 5–15% microglia, all of which influence nervous system development and maturation [1] (Fig. 8.1b). The most abundant types of glia in the brain are OLCs, including OPCs and mature oligodendrocytes.

8.1.2 GBM Development and Recurrence in the White Matter

Generally, GBM-enhanced mass lesions visualized by Gd-T1WI MRI are located in the white matter, through which GBM extensively invades [86]. Upon recurrence, enhanced mass lesions are identified in the white matter surrounding the empty post-resection cavity [9, 26] (Fig. 8.2a). Complete tumor resection was reported in 43 (48.3%) of 89 newly diagnosed patients with GBM, which was confirmed by Gd-T1WI MRI performed within 72 h after operation. After complete resection and chemo-radiotherapy, recurrence was observed in 30 (69.8%) cases in monthly MRIs during the observation period of

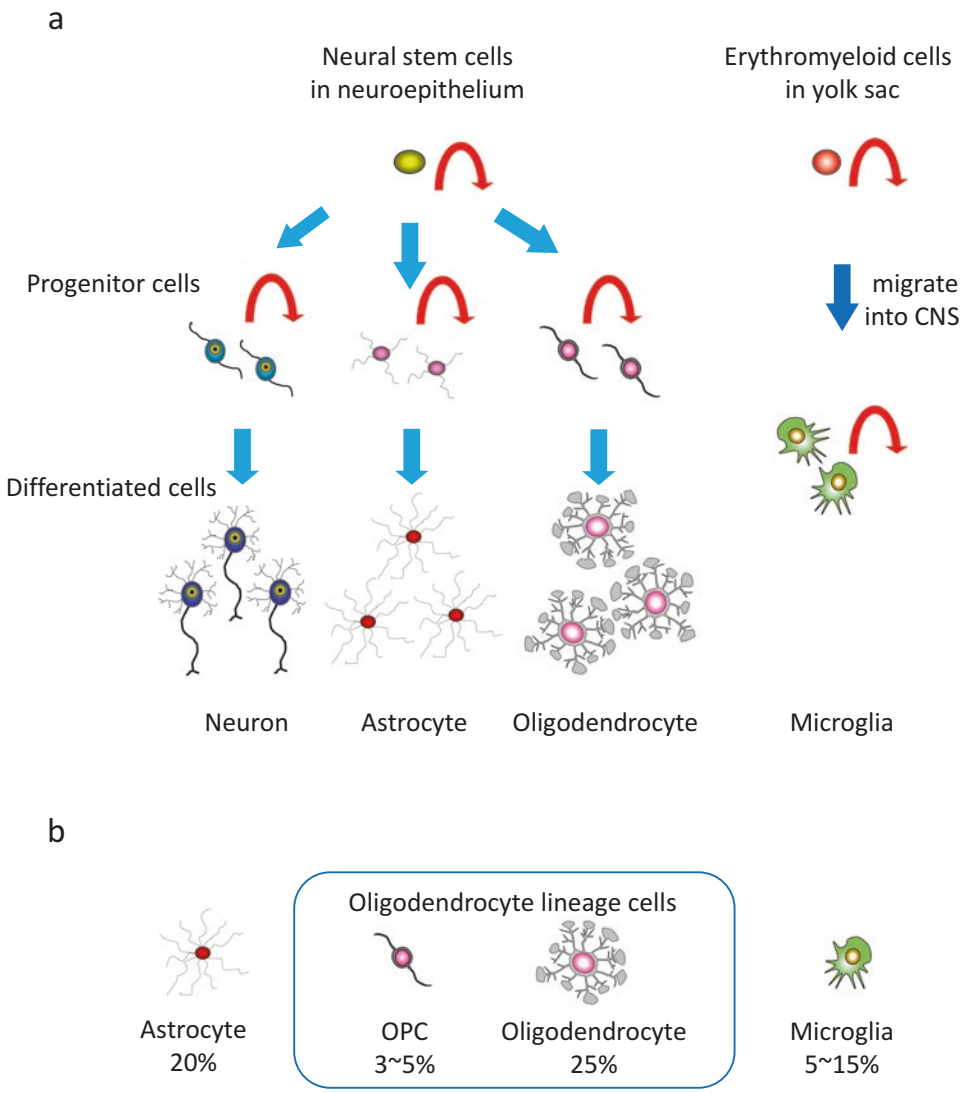

Fig. 8.1 Main cell populations of the brain. (**a**) Neurons, astrocytes, and oligodendrocytes differentiate from NSCs. However, microglia originate from erythromyeloid cells in the yolk sac and migrate into the CNS early during development. (**b**) OLCs are the most abundant cell type in the CNS

1.5–4.5 years post-resection. Primary recurrence was detected in the surrounding white matter in 26 (87%) cases and in the distant white matter in 1 (3%) case; dissemination was visualized in three (10%) cases, but recurrence in the gray matter was not observed [26] (Fig. 8.2b). These results suggest that white matter, but not gray matter, promotes the survival of GBM cells after chemo-radiotherapy. Thus, white matter at tumor borders provides factors that promote therapeutic resistance in GBM cells.

8.2 Change in miRNA Expression at the Tumor Border

To identify molecules at the tumor border involved in chemo-radioresistance and recurrence by promoting stem cell characteristics in GBM, miRNAs were evaluated because of their wide regulation of multiple targets and their secretion into the extracellular space, both which may alter the microenvironment [42, 44, 49].

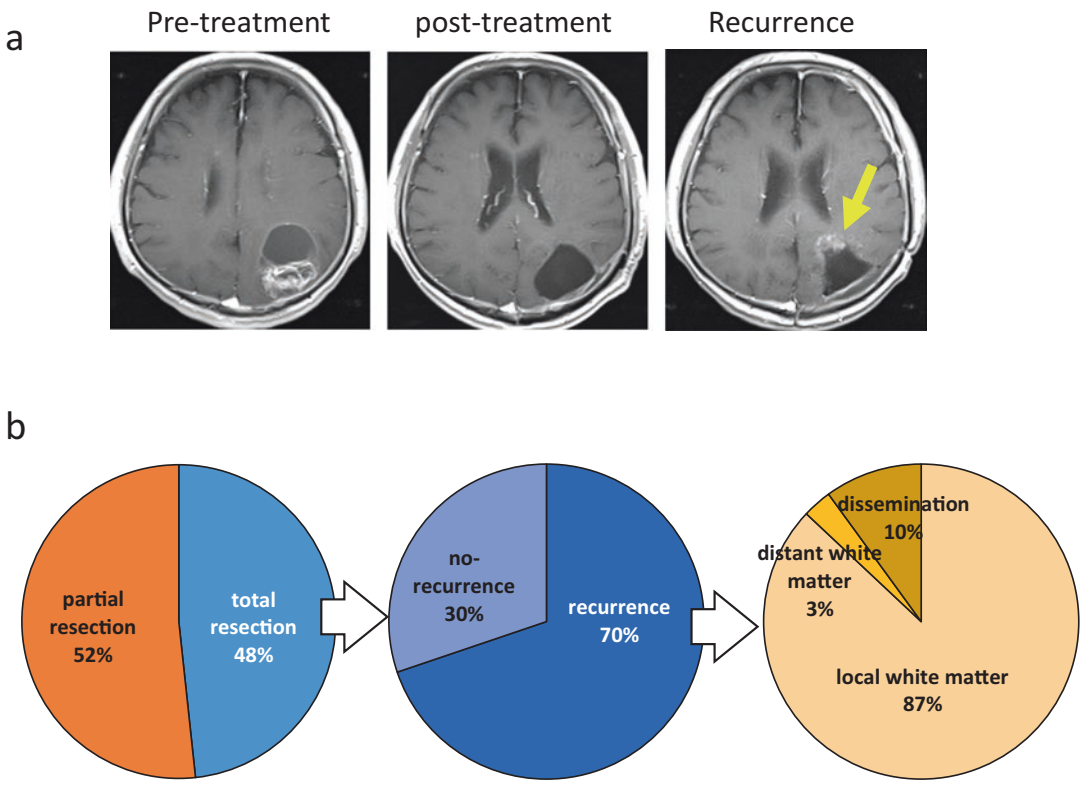

Fig. 8.2 GBM commonly recurs in the white matter. (**a**) Representative case of a patient with GBM post-treatment. Even after complete removal of the enhanced mass lesion and chemo-radiotherapy, recurrence is commonly observed in the white matter around tumor removal cavity (yellow arrow). (**b**) Complete removal of the enhanced mass lesion was achieved in 43 (48.3%) of 89 cases of newly diagnosed patients with GBM. After standard treatment, recurrence was detected in 30 cases (69.8%). Recurrence was seen in the local white matter in 26 cases (87%) and in the distant white matter in 1 case (3%), while dissemination was seen in 3 cases (10%)

To elucidate the features of this tumor border microenvironment, miRNA expression in resected tissue samples was compared from three sites in individual patients with GBM: the tumor mass (tumor), the border between the tumor mass and the brain where glioma and non-glioma cells co-exist (border), and the peripheral area distant from the tumor mass containing normal cells (periphery) (Fig. 8.3a). To obtain microarray data, tissue samples from three sites were divided in half: one half was used for pathological examination and the other half was used for purification of small RNAs if the pathological findings were suitable for downstream analysis [26] (Fig. 8.3b). miRNAs with altered expression were identified at the tumor border (Table 8.1).

8.2.1 Accumulation of Oligodendrocyte Lineage Cells (OLCs) at the Tumor Border

Interestingly, the top three miRNAs (*miR-219-5p*, *miR-219-2-3p*, and *miR-338-3p*) with increased expression at the tumor border play major roles in oligodendrocyte differentiation [3, 16, 17, 60, 89]. In miRNA in situ hybridization, increased *miR-219-5p*-positive cells were observed at the tumor border, but not within tumors. Immunohistochemical staining of the oligodendrocyte lineage markers Olig2, NG2 (also known as chondroitin sulfate proteoglycan 4), O4, and myelin basic protein (MBP) revealed increased

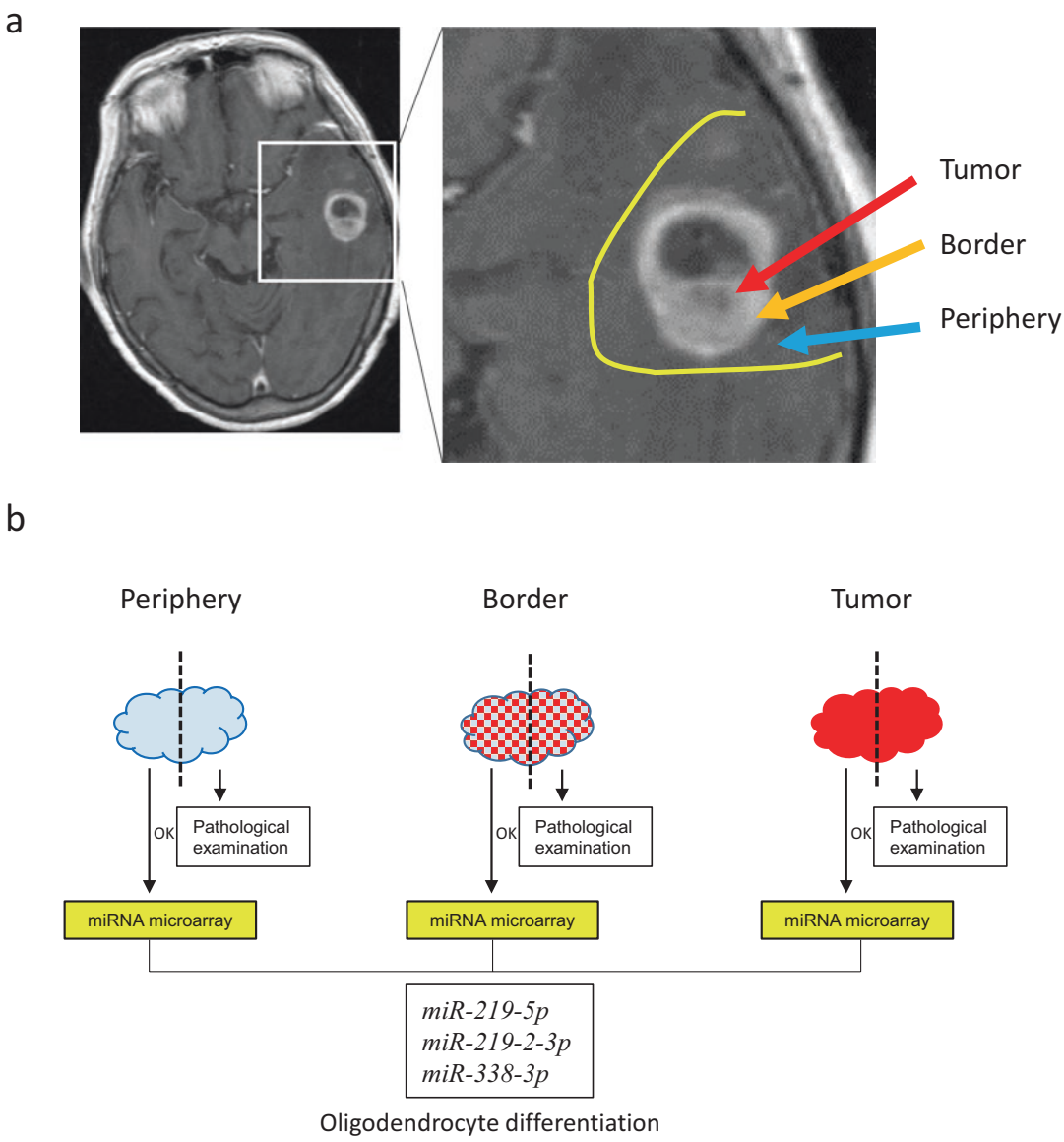

Fig. 8.3 miRNAs showing characteristically higher expression at the tumor border had functions related to oligodendrocyte differentiation. (**a**) The yellow line traces the tumor removal site. After tumor resection, three tissue samples were obtained from three regions (tumor, border, and periphery) and divided into two pieces. (**b**) Half of each piece was used for pathological examination. Pathologically, the tumor was defined as typical GBM tissue, the border as a mixture of tumor and normal cells, and the periphery as nearly normal brain tissue. The other half was used to purify small RNAs after pathological confirmation, and miRNA microarray analysis was done. The top three miRNAs (*miR-219-5p, miR-219-2-3p, and miR-338-3p*) that had increased expression at the tumor border had functions related to oligodendrocyte differentiation

marker-positive cells at the border [7, 26, 60, 85]. Upon pathological examination of 19 cases of newly diagnosed GBM samples containing the tumor border, abundant Olig2-positive cells within the tumor were found in ten (52.6%) cases but rarely in 9 (47.4%) cases [26]. In contrast, all cases showed accumulation of Olig2-positive cells at the tumor border [26]. NG2, O4, and

Table 8.1 miRNAs showing characteristically higher expression at the tumor border

miRNA	Periphery	Border	Tumor
Hsa-miR-219-5p	5.187	8.062	1
Hsa-miR-219-2-3p	5.845	8.037	1
Hsa-miR-338-3p	4.562	6.492	1
Hsa-miR-27b	1.491	2.176	1
Hsa-miR-23b	1.545	2.041	1

MBP were also detected at the border. These data suggest that OLCs, including OPCs, accumulate abundantly at the tumor border. However, accumulation of OLCs was only observed at sites where individual GBM cells invaded into the white matter, but not at the clear interface between the tumor and brain [26] (Fig. 8.4a).

8.2.2 Soluble Factors Secreted by OPCs Induce Stemness and Chemo-Radioresistance in GBM Cells

To investigate how OPCs interact with GBM cells, conditioned medium (CM) was prepared from the human A172 and T98G GBM cell lines (CM-A172 and CM-T98G), macrophages (CM-Mac), OPCs (CM-OPC), and OPCs plus macrophages (CM-OM). Interestingly, cell viability of OPCs was increased in medium containing CM-A172, CM-T98G, and CM-Mac [26]. This suggests that factors secreted from GBM cells directly affect the proliferation potential of normal OPCs (Fig. 8.4b). Further, addition of CM-OPC in the culture medium induced significantly higher expression of stemness genes *Nanog*, *Sox2*, aldehyde dehydrogenase isoform 1 (*ALDH1*), *Oct3/4*, and *Bmi1* and increased the sphere formation and cell viability of A172 cells [26]. Expression of ATP-binding cassette subfamily G member 2 (*ABCG2*), which plays a role in drug efflux, was significantly elevated in A172 cells cultured with CM-OPC. Addition of CM-OPC into the culture medium also increased the cell viability of A172 cells after treatment with temozolomide, the standard chemotherapy for GBM. Moreover, phosphorylated signal transducer and activator of transcription 3 (pSTAT3), which is important for radioresistance and stemness [36, 39, 43], was increased in A172 cells cultured with CM-OPC [26]. Thus, OPCs play an important role in GBM stemness and chemo-radioresistance [26] (Fig. 8.4b).

Moreover, DNA microarray analysis of OPCs and macrophages revealed increased expression of FGF1 and EGF in OPCs compared to in macrophages, and addition of FGF1 and EGF in the culture medium increased sphere formation and cell viability of GBM cells [26] (Fig. 8.4b). Recently, Kawashima et al. reported that CM-oligodendrocytes, established from human glioma tissue (WHO Grade II), increase the migration and invasion of GBM cells, in contrast to CM-fibroblasts established from GBM [41]. The authors concluded that these functions are regulated by angiopoietin-2 signaling [41] (Fig. 8.4b).

8.3 "Border Niche": A Novel Concept in GBM Characterized by Accumulation of OLCs

The perinecrotic niche (hypoxic niche) and perivascular niche within the tumor mass have been well studied to understand the mechanisms of stemness and chemo-radioresistance [11, 14, 31, 67, 68]. Despite complete removal of the enhanced mass lesion in Gd-T1WI, which removes these niches along with the tumor mass, recurrence commonly occurs in the white matter around the tumor removal cavity. At this site, OLCs including OPCs tend to accumulate, which promotes stemness and chemo-radioresistance in GBM cells. We defined this unique microenvironment outside of the tumor mass containing abundant OPCs as the "border niche," which promotes the survival and recurrence of GBM cells. This novel border niche is a new target of research and treatment [26, 28] (Fig. 8.4a).

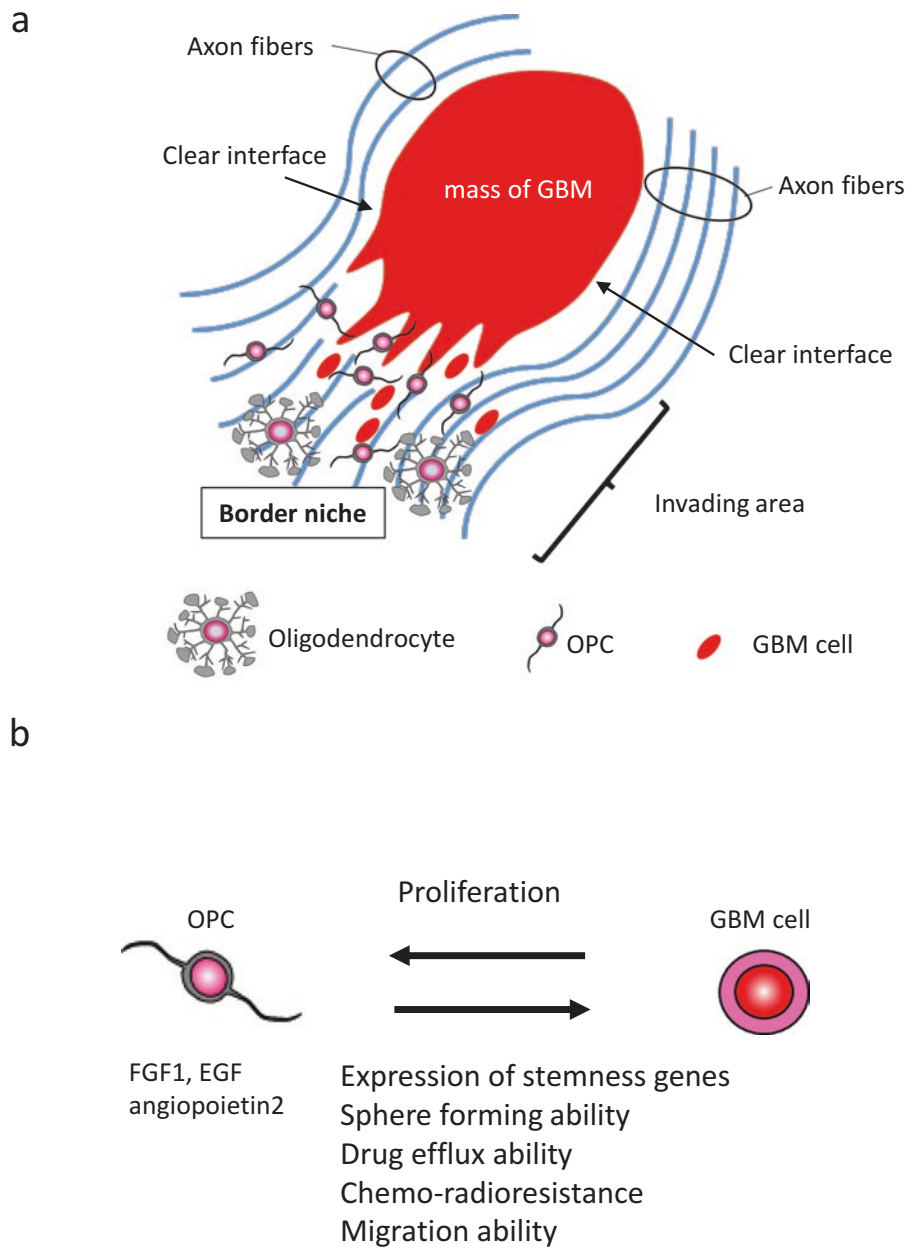

Fig. 8.4 OLCs, including OPCs, accumulate in the invading area. (**a**) Pathologically, the border between the tumor and brain was divided into two types: invading and clear interface areas. OLCs, including OPCs, accumulate in the invading area and form border niche with GBM cells. However, OLCs were not increased in the area showing clear interface. (**b**) GBM cells induce proliferation of OPCs. On the other hand, OPCs induce GBM cells with stem cell-like characteristics

8.3.1 OPCs Are Key Players in the Development and Invasion of GBM

OPCs are an important cell type in GBM and have been reported as the cells of origin for this tumor [21, 30, 51, 75]. Previously, we established artificial glioma-forming cells by overexpressing an active form of HRas in neural stem cells (NSCs), OPCs, and astrocytes isolated from the p53 knockout mouse. Interestingly, GBMs formed in the brains of nude mice after orthotopic injection of as few as ten cells from the NSC or OPC lines. However, cells originating from astrocytes required injections of 10,000 cells to form anaplastic astrocytoma, but never formed GBM. These results demonstrated that NSCs and OPCs have a similar potential to be the GBM cell of origin [29, 30].

Generally, rapid extension of GBM into the white matter, which is abundant in neurons and OLCs, is detected in Gd-T1WI MRI. One of the characteristic growth patterns associated with GBM is a butterfly shape due to invasion of GBM into the contralateral hemisphere through commissure fibers in the corpus callosum. Other patterns of extension are along the radiation of the corpus callosum, association fibers, or arcuate fasciculus in the bilateral hemispheres, and these patterns do not coincide with the vascular network. Because the axons are myelinated with oligodendrocytes, this location contains abundant proliferating OPCs [28]. Thus, GBM cells preferentially use myelinated axon fibers as a scaffold to migrate to and colonize additional tissue and construct the border niche to acquire stemness and therapeutic resistance [26, 28]. However, differentiated neurons cannot proliferate; therefore, GBM cells manipulate OPCs to form a tumor-supportive niche via the dynamic functions of OPCs in migration and proliferation. OPCs can promote the development, progression, invasion, resistance, and recurrence of GBM.

8.3.2 OPCs Dynamically Proliferate and Differentiate in Healthy Brains

Myelin, produced by differentiated oligodendrocytes, is a critical component of the vertebrate CNS. This myelination of axons regulates neuronal activities, mediates neural plasticity, and provides metabolic support [5, 20, 38]. Generally, the rate of myelin turnover is high, whereas the oligodendrocyte population itself is remarkably stable in the white matter [87]. Myelination and remyelination continue to occur throughout life [46]. OPCs constitute the majority of proliferating cells in the adult brain and exhibit specific characteristics, individual OPCs occupy their own territory, and OPC density is maintained through local proliferation. OPCs migrate rapidly to sites of injury [33] and are known to occupy regions of traumatic brain injury within one day post-injury [15]. Furthermore, they migrate and proliferate faster than astrocytes [18]. Neuronal activity also rapidly remodels white matter; for example, exercise stimulates OPC proliferation and oligodendrocyte differentiation within a few days [56].

Optogenetic, electrical, and pharmacogenetic stimulation of neurons induces oligodendrogenesis and myelination [22, 50, 58]. The selection of axons for myelination is strongly influenced by the relative activity of individual axons within a population [58]. In line with this observation, Bergles et al. reported that OPCs receive synaptic inputs from neurons [6], and neuron-oligodendroglial communication is mediated by glutamate and GABA in the CNS [25, 45].

However, not all axons are myelinated within the white matter tracts. For example, the proportion of unmyelinated fibers within the corpus callosum was relatively constant across species, with approximately 30% of fibers lacking myelination within the corpus callosum [61]. A study of the myelin distribution along single axons of pyramidal neurons revealed the distinct longitudinal distribution of myelin of individual

neurons [77]. Myelination does not peak in the human brain until the fifth decade, which then decreases rapidly starting at 60 years of age [52, 53]. Interestingly, decline in the ability of OPCs to myelinate axons coincides with the age most liable to develop GBM.

8.3.3 Heterogeneity of OPCs

OPCs exist in the various sites of the brain; however, their functional differences in these regions have not been well studied. OPCs in forebrain white matter (corpus callosum) have a shorter cell cycle (completed in ~10 days) than those in gray matter (motor cortex: ~36 days) of the mouse brain 60 days after birth [88]. Moreover, transplantation experiments revealed that OPCs from white matter differentiate into mature, myelinated oligodendrocytes preferentially in white matter compared to in gray matter, whereas gray matter-derived OPCs do so less efficiently [82]. Interestingly, OLCs have been classified into 13 populations with region- and age-specific distributions according to single-cell RNA sequencing data from 5072 cells [55], and Spitzer et al. reported that OPCs become regionally diverse and heterogeneous with age [73].

8.4 Other Supportive Cells

Several non-tumor cells, including microglia, macrophages, astrocytes, pericytes, and T cells, have been reported to play a pivotal role in promoting the proliferation, migration, and recurrence of GBM [11, 14, 67, 68]. Recently, it was reported that reciprocal signaling between GSCs and differentiated glioma cells promotes malignant progression [84].

8.4.1 Differentiated Glioma Cells

Differentiated glioblastoma cells (DGCs) express brain-derived neurotrophic factor (BDNF), whereas GSCs express the BDNF receptor NTRK2. DGCs communicate with GSCs through BDNF-NTRK2-VGF paracrine signaling to promote growth [84]. However, the microenvironments that foster this communication are within the tumor, not at the border, suggesting that DGCs have an important supportive function for GBM cells inside the tumor mass, but not at the border niche. Because DGCs do not seem to proliferate and migrate rapidly, they cannot quickly modulate the microenvironment at the border niche.

8.4.2 Microglia

From the perspective of oligodendrogenesis and myelination, microglia-derived factors can influence OLC chemoattraction, proliferation, differentiation, and myelination/remyelination. Moreover, microglia enhance the differentiation of neural stem/progenitor cells into OLCs [10, 57, 69]. In GBM tissue, bone marrow-derived macrophages are prominent in the perivascular areas, whereas resident microglia are present in high numbers in the peritumoral region [12, 13]. Because the border niche exists in the peritumoral region where abnormal vessels have not yet developed sufficiently, microglia constitute the majority of glioma-associated microglia/macrophages at the border. Further investigation into the interaction between OPCs and microglia is needed to reveal the mechanisms of the border niche in GBM progression and recurrence [28].

8.4.3 Astrocytes

The identity of astrocyte lineage cells remains unclear. Interestingly, subpopulations of healthy astrocytes in the adult brain and their glioma counterparts are endowed with diverse cellular, molecular, and functional properties. Further, some populations contribute to synaptogenesis and tumor pathophysiology [37]. Astrocytes in the tumor microenvironment promote the proliferation, migration, and therapeutic resistance of GBM cells [8, 24]. Interestingly, glioma-associated astrocytes (tumor-associated astro-

cytes) show a different miRNA expression profile from normal astrocytes [40]. Based on the supportive function of oligodendrogenesis, astrocytes affect the proliferation and remyelination of OPCs [54, 59] and therefore play indirect roles in forming the border niche. Astrocytes have a low proliferation rate and low migration potential to sites of wound injury [4], whereas OPCs and microglia play an immediate role in CNS injury [18]. These data suggest that OPCs and microglia play a more critical role in border niche formation than astrocytes [26, 28].

8.4.4 Neurons

Neuronal activity not only affects the migration and proliferation of OPCs [20, 22, 50, 58] but also promotes the survival of GBM cells directly. Neuronal regulation of glioma is dependent on the cleavage and secretion of the synaptic adhesion molecule neuroligin-3, which promotes glioma proliferation through the PI3K-mTOR pathway [79, 81] (Fig. 8.5a).

Seizure is one of the accompanying symptoms in patients with glioma. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors (AMPARs) mediate neurotransmission in excitatory synapses and are expressed not only in neuron and glia cells but also in GBM cells [35]. Inactivation of AMPARs suppresses migration and induces apoptosis in glioma cells [35] (Fig. 8.5b).

Moreover, some GBM cells form synapses with neurons, and then synaptic and electrical integration into neural circuits promotes glioma progression [80]. Recently, perampanel (AMPAR inhibitor) was used as an anticonvulsant. In in vivo experiments, an approximately 50% decrease in glioma proliferation was observed in perampanel-treated mice compared to in vehicle-treated control mice [80]. Additionally, glutamatergic synaptic input to glioma cells drives the progression of glioma, and blockade of neuroglioma synapses-driven synaptic communication between neurons and GBM cells via genetic and pharmacological blockade of AMPAR signaling reduced GBM cell malignancy, leading to attenuated glioma progression [78]. Thus, these results showing direct interactions between neurons and GBM cells provide insight into progression and niche formation in GBM (Fig. 8.5a, b).

8.5 Further Perspective

Neuronal activity promotes the progression of GBM and proliferation of OPCs [22, 50, 58, 78, 80]. However, various aspects of this process remain unresolved. The soma of the neuron is located in the gray matter and the axon in the white matter. Generally, synapses exist in the gray matter. OPCs in the white matter show a higher potential for proliferation than those in the gray matter [55, 73, 82, 88]. However, GBM develops and recurs in the white matter. In the white matter, neurons, GBM cells, and OPCs may interact directly in a synaptic and non-synaptic manner, or intervention of OPCs between neuron and GBM cells occurs to promote the progression of GBM. Further studies are needed to reveal the mechanisms of invasion, proliferation, chemo-radioresistance, and recurrence of GBM (Fig. 8.6).

8.6 Conclusion

The ultimate goal of GBM treatment is to completely abolish GBM cells. Standard treatment for patients with GBM is maximal safe resection and chemo-radiotherapy to inhibit recurrence and dissemination. GBM cells rapidly accumulate mutations, making the tumor highly heterogeneous [63, 72]. The application of therapies targeting not only GBM cells but also non-glioma cells, OPCs, neurons, microglia, and other cells that form the border niche will contribute to better prognosis [26, 28] (Fig. 8.6).

Further studies of the border niche may provide insight into fundamental processes such as the development, progression, migration, and recurrence of GBM and may be useful for preventing recurrence in patients.

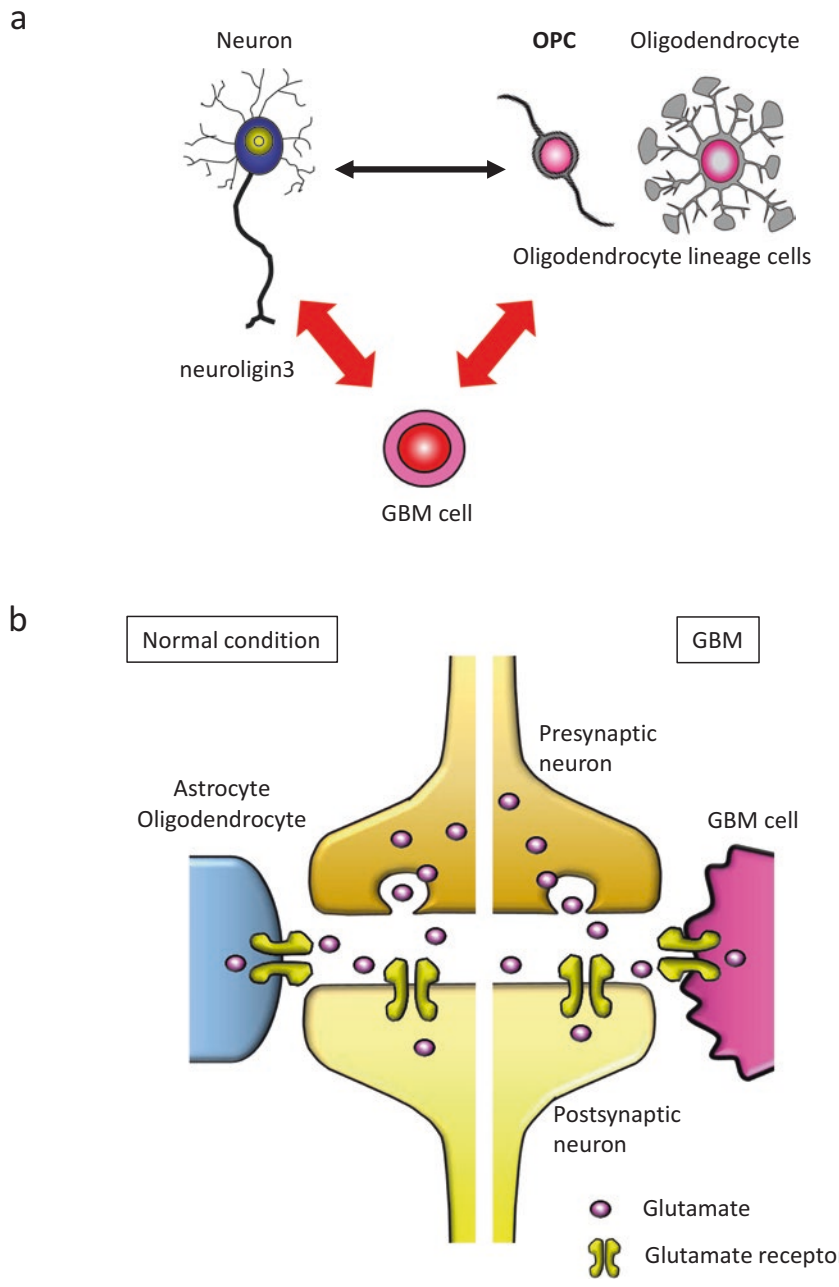

Fig. 8.5 Neurons interact with glial cells and GBM cells. (**a**) Neurons interact closely and dynamically with OLCs, including OPCs. The direct interaction between neurons and GBM cells has recently been discussed. Understanding the mechanisms of interaction among neurons, OPCs, and GBM cells is crucial for improving the prognosis of GBM patients. (**b**) Glutamate receptors are expressed on neurons, astrocytes, oligodendrocyte, and GBM cells. Signals from neurons promote proliferation and migration of GBM cells

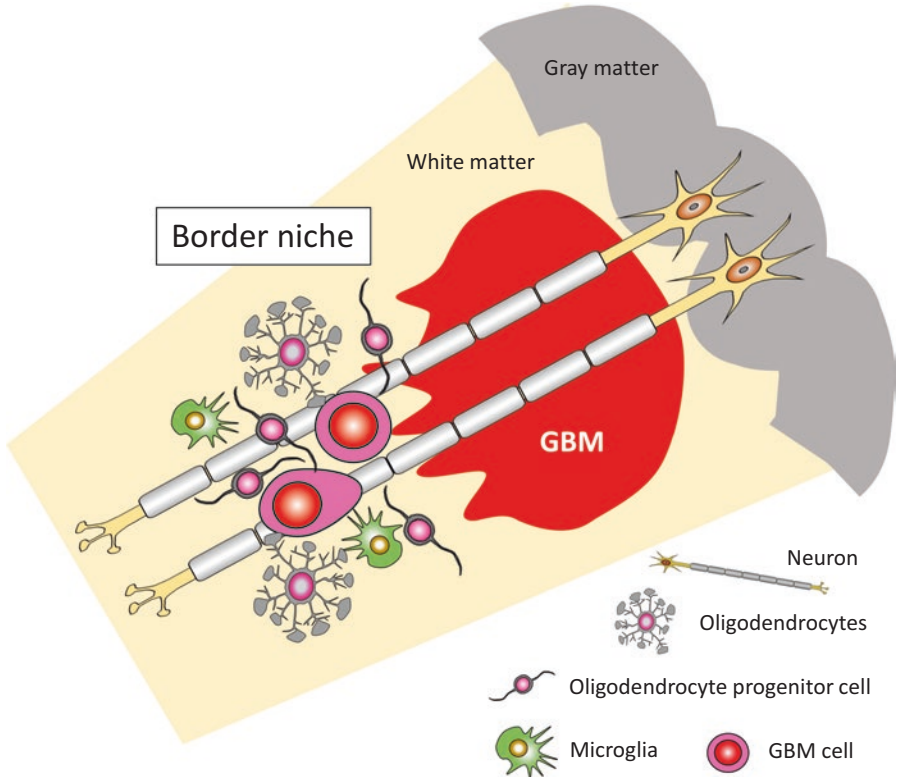

Fig. 8.6 Border niche in GBM. In the border niche, crosstalk between GBM cells and non-GBM cells, OPCs, and microglia promotes stemness and therapeutic resistance in GBM cells. Neuronal activity induces proliferation of both OPCs and GBM cells. GBM cells prefer to migrate within the fasciculus of axons where abundant OLCs, including OPCs, exist, particularly at the border. The border niche is characterized by GAOs

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1234, https://doi.org/10.1007/978-3-030-37184-5