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Alexander Birbrair *Editor*

Tumor Microenvironment

Molecular Players – Part B

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

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

Preface

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

This book *Tumor Microenvironment: Molecular Players – Part B* presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different key molecular players within the tumor microenvironment during cancer development. Further insights into the 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 organs using state-of-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different tissues. 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 different molecular players within the tumor microenvironment in various tissues. Ten chapters written by experts in the field summarize the present knowledge about distinct characteristics of the tumor microenvironment during cancer development.

Jörg H. Leupold and colleagues from the University of Heidelberg discuss MicroRNAs in the tumor microenvironment. Laura P. Stabile and colleagues from the University of Pittsburgh update us on the impact of estrogen in the tumor microenvironment. Peter A van Dam and colleagues from the University of Antwerp describe the non-bone related role of RANK/RANKL signaling in cancer. Hyo-Jin Yoon and Young-Joon Surh from Seoul National University summarize current knowledge on modulation of cancer cell growth and progression by Caveolin-1 in the tumor microenvironment. Kishore B. Challagundla and colleagues from the University of Nebraska Medical Center address the importance of exosomes, as novel players of therapy resistance in neuroblastoma. Ying-Ting Zhu and colleagues from Tissue

Tech, Inc. compile our understanding of COX-2 signaling in the tumor microenvironment. Yutaka Kawakami and colleagues from Keio University School of Medicine focus on the renin–angiotensin system in the tumor microenvironment. Juan Antonio Marchal and colleagues from the University of Granada give an overview of stem cell secreted factors in the tumor microenvironment. Sophie Sibéril and colleagues from Sorbonne University present the tight interplay between therapeutic monoclonal antibodies and the tumor microenvironment in cancer therapy. Finally, Himanshu Arora and colleagues from the University of Miami talk about nitric oxide within the tumor microenvironment.

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

Belo Horizonte, MG, Brazil — Alexander Birbrair

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1 MicroRNAs in the Tumor Microenvironment

Nitin Patil, Heike Allgayer, and Jörg H. Leupold

Abstract

The tumor microenvironment (TME) is decisive for the eradication or survival of any tumor mass. Moreover, it plays a pivotal role for metastasis and for providing the metastatic niche. The TME offers special physiological conditions and is composed of, for example, surrounding blood vessels, the extracellular matrix (ECM), diverse signaling molecules, exosomes and several cell types including, but not being limited to, infiltrated immune cells, cancer-associated endothelial cells (CAEs), and cancer-associated fibroblasts (CAFs). These cells can additionally and significantly contribute to tumor and metastasis progression, especially also by acting via their own deregulated micro (mi) RNA expression or activity. Thus, miRNAs are essential players in the crosstalk between cancer cells and the TME. MiRNAs are small non-coding (nc) RNAs that typically inhibit translation and stability of messenger (m) RNAs, thus being able to regulate several cell functions including proliferation, migration, differentiation, survival, invasion, and several steps of the metastatic cascade. The dynamic interplay between miRNAs in different cell types or organelles such as exosomes, ECM macromolecules, and the TME plays critical roles in many aspects of cancer development. This chapter aims to give an overview on the multiple contributions of miRNAs as players within the TME, to summarize the role of miRNAs in the crosstalk between different cell populations found within the TME, and to illustrate how they act on tumorigenesis and the behavior of cells in the TME context. Lastly, the potential clinical utility of miRNAs for cancer therapy is discussed.

Keywords

miRNAs · Tumor microenvironment · Metastasis · CAF · TAM · TIDC · T-reg · Hypoxia · CD4^{+} · CD8^{+} · NK · Cancer · Immune checkpoint · Exosomes · ECM

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1.1 Introduction

In the early nineties of the last century, Victor Ambros and his colleagues discovered the first miRNA in *C. elegans*, essential for the embryonic development of this organism [1]. Their discovery led to the surprising conclusion that the expression of a gene, *lin-14*, was not regulated by a protein, but through a short RNA, which appeared to bind within the 3′- untranslated region (UTR) of *lin-14* mRNA, suggesting a new posttranscriptional regulation via antisense RNA-RNA interaction. In a few years, thousands of miRNAs were described and it became clear that this novel translational control mediated by ribonucleic acids has impacted virtually every biological process and changed our thinking about gene regulation [2].

Generally, miRNAs belong to the family of endogenously expressed ncRNA molecules and are defined by their length of approximately 18–25 nucleotides. They are evolutionarily conserved and nearly 3000 functionally diverse miRNAs have been identified until now [3]. Most of miRNA genes are located within the human genome in intragenic regions, UTRs, and repeat regions of the genome [4]. Here, they are often found as clusters from where they are transcribed as discrete polycistronic transcripts, or they share the promoter of host genes and get spliced from their mRNA transcripts during biogenesis [5]. MiRNA genes are first transcribed into the primary miRNA (pri-miRNA) by RNA polymerase II (Pol II). Thereafter, these typically 1-kb-long primary transcripts, containing the actual miRNA sequence, undergo diverse nuclear processing steps of maturation with the participation of the proteins Drosha and DiGeorge critical region 8 (DGCR8). The resulting miRNA precursor (pre) miRNA is exported into the cytoplasm with the help of Exportin 5, together with the guanosine-5′-triphosphate ras-related GTP-binding nuclear protein (RAN). After release, the pre-miRNA is cleaved by the ribonuclease (RNase) III enzyme Dicer, leading to the mature miRNA duplex. Finally, this duplex is loaded onto an Argonaut (ARGO) protein to form the effector complex called RNA-induced silencing complex (RISC) where it binds seed sequences within 3´-UTRs of specific mRNAs to mediate their degradation, destabilization, or translational inhibition [6]. Interestingly, latest research in this field showed that the expression of certain circular (circ) RNAs plays an important role in the regulation of miRNA, by acting as miRNAs sponges through abundant binding sites for microRNAs to modulate the activity on their target genes, representing a new layer of gene control by ncRNAs [7].

Based on the complexity of the above-described pathway and numerous auxiliary regulatory factors found up to date to be involved in successful miRNA biogenesis, a tight regulation is needed at multiple levels to prevent aberrant miRNA gene transcription in the cells. This is even more important given the notion that more than 60% of human protein-coding genes contain at least one conserved miRNA seed sequence, and that any deregulation of miRNAs is most likely associated with human disease, particularly cancer [8]. In consequence, miRNAs can exert tumor suppressive or oncogenic functions and affect, directly or indirectly, tumor progression and metastasis because the misdirected transcription of miRNAs, or mutations within the seed sequences, leads to aberrantly expressed proteins [9].

During the last decades, cancer research was mainly focused at malignant cells to understand the process and driving forces, which transform a normal cell a cancer cell and to cancer cells with metastatic capacity. However, to understand the whole process of how cells get transformed and can survive or metastasize into different tissues and organs, it is necessary to realize that cancer cells are not the only players necessary to manifest the disease. Since many years, pioneered by Isaac Witz and others [10], it became very clear that tumors strongly depend on external signals from their microenvironment, leading to the concept of the TME as one of the essential factors to promote disease progression, local resistance, immune-escape, and metastasis of tumor cells [11].

Generally, the TME is defined as the environment around a tumor within the tissue in which cancer cells are embedded. Together with other cells such as stroma and endothelial cells, ECM, infiltrating cells such as macrophages and lympho-

cytes, soluble products, namely growth factors, cytokines and antibodies, proteases, as well as other types of enzymes it resembles a network between a variety of cells with non-cellular components [12]. Interestingly, non-neoplastic cells can account for more than 50% of the total tumor mass and produce and release cytokines, chemokines, growth factors, matrix remodeling enzymes, vesicles, and other soluble factors into the tumor mass, often supporting tumor growth [13]. Moreover, during tumor development, remodeling of this tissue occurs through the modification of the physical scaffold and structural framework provided by the extracellular matrix (ECM). This complex network of collagen and fibronectin fibrils associated with glycoproteins, proteoglycans, and polysaccharides, also provides biochemical signals by hosting growth factors and chemokines modulating tumor cell growth, migration, and metastasis [14].

Due to their responsibility to form the ECM, specifically CAFs are found in the TME and play a pivotal tumor-promoting role [15]. Besides other stromal cell types like pericytes, vascular endothelial cells and cancer-associated adipocytes are present [16]. Together with the ECM, these stromal cell types and adipocytes are able to support tumor growth by providing paracrine and juxtacrine signaling molecules like hepatocyte growth factor (HGF), fibroblast growth factor (FGF), the chemokines C-C motif ligand (CCL) 5 and CCL2, and the inflammatory factors interleukin (IL)-6, and tumor necrosis factor (TNF)-α, which further promote the proliferation and invasion of tumor cells and the formation of neovascularization [17, 18]. Additionally, the TME is characterized by the presence of diverse tumor-infiltrating immune cells as a consequence of inflammation, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory Thymus (T)-cells (Treg), dendritic cells (DC), as well as effector immune cells such as T-lymphocytes and natural killer (NK) cells [19]. Unfortunately, the TME can provide an immunosuppressive environment, which blocks antitumor immunity to a large extent. Typically, NK- and T-cell cytotoxic activities are suppressed, T-cell proliferation is inhibited, and expression of major histocompatibility complex (MHC) molecules is downregulated, thereby precluding long-standing protective immunity and allowing tumor cells to escape attacks from the immune system [20]. On the sub-cellular level, extracellular vesicles, predominantly exosomes, are critical vehicles within the TME for intercellular communication [21]. These vesicles traffic between the different cell types found in the TME and specifically cancer-associated exosomes containing miRNAs can promote tumor survival and growth. Thus, they contribute to establishing tumorigenic niches, for example, by inducing angiogenesis, remodeling of the ECM, and impairing the function of immune cells [22]. Finally, due to the rapid growth and abnormal structure of the TME, most cells in a solid tumor mass suffer from exposure to hypoxia conditions. The generated hypoxic signaling, a discovery recently awarded with the Nobel Prize in Physiology or Medicine 2019, is mainly mediated by hypoxia-inducible transcription factors (HIFs), which in turn induce expression changes of genes involved in angiogenesis, growth, and cell survival [12].

Taken together, cellular and sub-cellular components of the TME are recognized to play a pivotal role to regulate and support cancer development and metastasis. The discovery that miRNA dysregulation substantially impacts TME-associated processes sheds new light on understanding cancer proliferation, angiogenesis, and metastasis through interactions between malignant cells, stromal cells, immune cells, and non-cellular components in the TME. Therefore, this chapter aims to summarize the versatile effects of miRNAs on the complex interplay between the different components of the TME and to offer a momentary overview of this rapidly moving field. Finally, the therapeutic potential of miRNAs to overcome the limitations of checkpoint blockage is discussed as an example of how pre-clinical knowledge on TME-based interactions can be transferred to potentially improve future immune therapies.

1.2 Hypoxia as Modulator for miRNA Expression in the TME

Solid cancers and their TME are commonly characterized by hypoxic conditions because excessive cancer cell proliferation, but also growth of the complete tumor mass above a critical size, results in deprivation of oxygen due to insufficient blood supply from abnormal tumor microvasculature. Especially in the center of metastatic lesions, hypoxic central areas can often be visualized in computed tomography (CT) or magnetic resonance imaging (MRI) scans. As a result of this condition, a specific hypoxia signaling is induced at the cellular level to restore oxygenation and to minimize the negative effects of the hypoxic environment [23–25]. This adaptive response connected to changes in tissue oxygenation is mediated by a heterodimeric protein that consists of two proteins, hypoxia-induced factor (HIF)-1α and HIF-1β, which together constitute HIF-1. The HIF-1β subunit is constitutively expressed, whereas the HIF-1α subunit expression is tightly regulated, depending on the oxygen status in the cell [24–26]. Once induced, the two subunits dimerize and bind to *cis*-acting hypoxia response elements at promoters that contain HIF-1 binding sites. Following this process, numerous miRNA genes are induced in response to hypoxic stress and HIF stabilization in the TME [27]. A diagrammatic representation of how hypoxia modulates miRNA expression in the TME is shown in Fig. 1.1.

The first study providing the functional link between hypoxia and miRNA expression came from Kulshreshtha et al. [28]. In this groundbreaking work, the authors aimed to characterize molecular mechanisms responsible for the hypoxic survival of neoplastic cells and, in a panel of human cancer cell lines, found a specific hypoxia-regulated miRNA profile of 23 mature miRNAs. Since the vast majority of these hypoxia-regulated miRNAs (hypoxymiRNAs) were also found overexpressed in diverse types of tumor, these findings strengthen the theory that hypoxia displays a key trigger for miRNA alterations in cancer [28]. Subsequently, multiple studies not only confirmed these signatures but also described more miRNAs found to be upregulated under hypoxic conditions in various solid cancer entities like lung, liver, bladder, gastric, pancreatic, or cervical tumors [29–34].

Among them, one of the best-studied hypoxymiRNAs is miRNA-210, and various groups have demonstrated that miRNA-210 overexpression is induced by HIF-1 and acts on a number of targets regulating carcinogenesis, angiogenesis, cell proliferation, and apoptosis [35, 36]. Additionally, miRNA-210 directly targets the myelocytoma (Myc)/Myc-associated factor X (Max) transcriptional network, which is essential in many cancer pathways and mediates the adaptation of cancer cells to hypoxic conditions by favoring growth and survival through increasing Myc activity [37]. Hypoxia-induced miRNA-210 can also increase proliferation and migration via targeting iron-sulfur cluster scaffold homolog 2 (ISCU2) and protein tyrosine phosphatase non-receptor types (PTPN) 1 and 2 [38, 39]. Moreover, miRNA-210 has been reported to promote migration and invasion capability of hepatocellular carcinoma by directly targeting vacuole membrane protein 1 (VMP1) [40]. Besides miRNA-210, very similar abundant expression under the regulation of HIF-1 was quickly demonstrated for miRNA-21 [33]. Most obviously, miRNA-21 overexpression induces tumor angiogenesis under this condition, through targeting PTEN, leading to the activation of serine/threonine protein kinase B, also known as AKT, and extracellular-signal-regulated kinase (ERK) 1/2 signaling pathways, thereby enhancing vascular endothelial growth factor (VEGF) expression and also HIF-1α [41]. In this context, it was also demonstrated that miRNA-21 expression is increased in exosomes derived from hypoxia-induced cells and can promote chemoresistance to normoxic cells [42].

Beside these most abundant hypoximiRNAs, diverse studies during the last years have confirmed the signature found by Kulshreshtha et al. and additionally identified more miRNAs and their potential targets in the hypoxia context. For example, for pancreatic cancer, it was shown that clinicopathological characteristics and prognosis

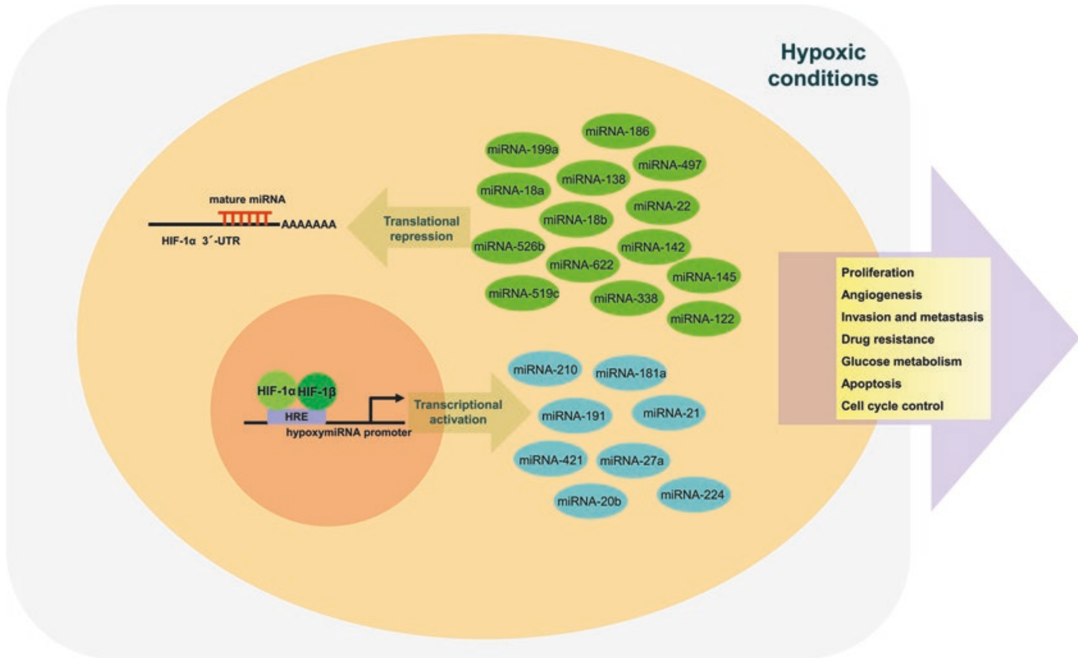

Fig. 1.1 Hypoxia as a modulator for miRNA expression in the TME. Hypoxic conditions lead to increased dimerization of HIF-1α with HIF-1β and binding to HRE elements within the promoter region of hypoxia-inducible miRNA genes. In turn, HIF-1 α mRNA translation is controlled by diverse miRNAs. This complex regulatory network between miRNAs/HIF-1α plays a critical role in tumorigenicity by targeting pathways controlling cell proliferation, the cell cycle, metabolism, angiogenesis, drug resistance, or invasion, migration, and metastasis

are closely associated with the expression of miRNA-191, whose expression is regulated by HIF-1 [43]. Additionally, it soon became obvious that HIF-1-induced miRNA-191 expression is significantly inducing angiogenesis, proliferation, migration, and metastasis in breast cancer, non-small-cell lung cancer (NSCLC), and transformed human bronchial epithelial cells (HBE) [29, 44, 45]. In this context, miRNA-191 was shown to be a critical regulator of transforming growth factor (TGF)-β signaling and promotes cell migration by inducing TGF-β2 expression under hypoxia. This can happen either by direct binding or indirectly by regulating levels of a RNA-binding protein, human antigen R (HuR). Moreover, the levels of several TGF-β pathway genes, like VEGF-A, SMAD (an acronym from the fusion of *Caenorhabditis elegans Sma* genes and the *Drosophila Mad*, Mothers against decapentaplegic) family member 3 (SMAD3), connective tissue growth factor (CTGF), and bone morphogenetic protein (BMP) 4, were found to be higher in miRNA-191 overexpressing cells, and anti-miRNA-191 treatment given to breast tumor spheroids led to drastic reduction in spheroid tumor volume [44]. Similarly, another study had identified the transcription factor nuclear factor 1A (NFIA) as a direct target of miRNA-191 under chronic hypoxic conditions, being responsible for promoting proliferation and migration [29]. Other examples of aberrant miRNAs expression explained by HIF-1 induction are miRNA-224, miRNA 421, and miRNA-27a. For example, miRNA-224 expression depends on HIF-1 in pancreatic ductal adenocarcinoma (PDAC) cells and tissue, which is related to migration and proliferation [46]. Additionally, diverse groups demonstrated that miRNA-224 and miRNA-421 promote gastric cancer cell growth, migration and invasion, and identified a Ras association domain tumor suppressor protein (RASSF) 8, epithelial (E)-Cadherin and caspase-3 as direct targets under hypoxic conditions [30, 47]. The first hint that miRNAs in the miRNA23a/27a/24 cluster play a

pivotal role in hypoxia came from a study on the angiogenic factor with G-patch and FHA domain (AGGF) 1 in high-grade urothelial carcinoma. Here, Xu et al. were able to prove that the hypoxia-induced decrease of AGGF1 is directly connected to a HIF-1-mediated induction of miRNA-27a expression [48]. Subsequently, two independent groups investigated pathways responsible for drug resistance in gastric cancer and concluded that HIF-1 induces chemoresistance, besides miRNA-181a and miRNA-20b, via miRNA-27a through targeting multidrug resistance genes [49, 50]. Later, another group found that all three miRNAs in the miRNA23a/27a/24 cluster are the most abundantly upregulated cluster miRNAs in colorectal cancer and that they collectively regulate the glucose metabolic network through regulating various metabolic pathways and targeting multiple tricarboxylic acid cycle (TCA)-related genes [51].

In this context, it became soon obvious that the crosstalk between miRNAs and HIF-1α is not only characterized by transcriptional upregulation of specific miRNAs. In addition, TME-related miRNAs can also modulate the activity of HIF-1α, finally exerting not only pro- but also anti-tumor effects on tumor cells [52]. One of the first studies on translational repression and HIF-1α came from Weiwei et al. [53]. Here, the authors investigated the co-expression of miRNA-199 and HIF-1α in prostate cancer and demonstrated a negative correlation between these two molecules based on the direct binding of miRNA-199 within the 3′-UTR of HIF-1α. This finding was further supported by multiple studies investigating the role of the miRNA-199 family in this regard. For example, it was shown that enforced expression of miR-199a-5p led to down-modulated expression of HIF-1α as well as of other pro-angiogenic factors such as VEGF-A, IL-8, and fibroblast growth factor (FGF) 2 in hypoxic multiple myeloma (MM) cells in vitro [54]. Furthermore, these data were also supported by Yang et al. [55] in melanoma cell lines, whereby overexpression of miRNA-199a-5p suppressed cell proliferation and arrested the cell cycle in the G1 phase. Moreover, in vivo overexpression of miRNA-199a-5p significantly inhibited xenograft growth and downregulated the expression of HIF-1α. Increased levels of miRNA-138 can significantly inhibit the expression of HIF-1α in renal cell carcinoma, increase apoptosis, and reduce the migratory potential of these cancer cells [56]. Besides, miRNA-138 suppresses cell invasion and metastasis in ovarian cancer and malignant melanoma cells by targeting HIF-1α together with SRY-related high mobility group box 4 (SOX4) [57, 58]. Two members of the miRNA-18 family have also been found to play a crucial role in the TME by suppressing HIF-1α expression. Several groups showed that miRNA-18a regulates apoptosis and invasion of gastric cancer via HIF-1 suppression, increased radio-sensitivity for lung cancer cells, and reduced metastasis of breast cancer [59–61]. Similarly, miRNA-18b inhibits the growth of MM cells in vitro and in vivo by directly targeting the 3´-UTR of HIF-1α [62]. It is worth noticing that besides these miRNAs, HIF-1α was also confirmed as a common target gene for multiple other miRNAs during the last years. Investigations on colon cancer revealed miRNA-526a and miRNA-22 to inhibit key steps in cancer development by targeting HIF-1α [63, 64]. Similar effects were observed in breast and lung cancer for miRNA-497, miRNA-622, and miRNA-519c [65–67]. In line with the context, other groups identified HIF-1α as a target of miRNA-142, miRNA-186, miRNA-122, miRNA 145, or miRNA-338 in pancreatic and gastric cancers as well as hepatocellular carcinoma (HCC) and nasopharyngeal carcinoma (NPC) [68–72].

Finally, the hypoxic conditions do not only affect HIF-1 induction and upregulation of miRNA expression, but can also be one driving force to mediate the repression of miRNA biogenesis proteins, like Drosha, Dicer, exportin (XPO) 5, DGCR8, TCR gamma alternate reading frame (TARP) 2, or AGO [73, 74]. An observation that hypoxia represses miRNA biogenesis by affecting the expression of specific proteins involved in this process came from studies of Bandara et al., where it was shown that cancer cells lead to a significant reduction of Dicer, Drosha, TARBP2, and DCGR8 expression [75]. Moreover, other studies demonstrated that Drosha and Dicer are independent pre-

dictors of cancer patient outcome and cancer progression, and that the loss of these molecules in the cells directly impacts tumor development and patient survival [76–78]. Additionally, a comprehensive study using reverse transcription quantitative polymerase chain reaction (RT-QPCR) had investigated 19 genes specifically involved in miRNA biogenesis in several hundred colorectal cancer tissues and concluded that, besides Drosha, a clear correlation of TARBP2, XPO5, trinucleotide repeat containing adaptor (TNRC) 6A, and DEAD-box helicase (DDX) 17 with survival and prognosis of these patients was observed [79]. Possible mechanisms to regulate the expression of these molecules were found by other studies, including hypoxia-induced epigenetic regulators or hypoxia-mediated binding of specific transcription factors to change the promoter activity of these genes [80–82]. Additionally, a study has shown that epidermal growth factor receptor (EGFR) suppresses the maturation of specific tumor-suppressor-like miRNAs in response to hypoxic stress through phosphorylation of AGO2. The association between EGFR and AGO2 is enhanced by hypoxia, leading to elevated AGO2 phosphorylation, which in turn reduces the binding of Dicer to AGO2 and inhibits miRNA processing from precursor miRNAs to mature miRNAs [83]. Alternatively, miRNA biogenesis proteins are by themselves targeted by the translational control through diverse hypoxia-induced miRNAs. In line with this concept, a study has shown that miRNA-630, which is upregulated under hypoxic conditions, targets and downregulates Drosha and Dicer [84]. Other studies identified miRNA-107 and miRNA-122 as direct translational regulators of Dicer, thereby promoting metastasis and neovascularization [85, 86].

1.3 Exosomal miRNAs, Dysregulated miRNAs, and CAFs

Among all the stromal cells present in the TME, CAFs are one of the most abundant and critical components of the tumor mesenchyme, which not only provide physical support for tumor cells but also play a key role in promoting and retarding tumorigenesis [87]. Normally, the primary role of activated fibroblasts is to remodel and regenerate tissues in a highly regulated manner. In consequence, these normal fibroblasts only transiently acquire activity; otherwise, pathological conditions such as tissue fibrosis and chronical inflammation can arise [88]. During tumor development, this indispensable ability can be hijacked, leading to chronic tissue repair by the generation of distinct, misregulated, tumor-promoting fibroblasts, termed CAFs [89]. After transformation, CAFs have been shown to exert multiple effects on cancer progression, including the regulation of cancer growth, angiogenesis, metastasis, metabolism changes, or remodeling of the tumor microenvironment [90]. During this process, CAFs and cancer cells typically regulate each other by secreting exosomes containing a variety of bioactive molecules, including miRNAs, DNAs, RNAs, and proteins. Not surprisingly, a growing number of publications during the past years have shed light on this specific interaction between exosomal miRNAs, miRNA dysregulation, CAF formation, and activation in the TME [87]. Another crucial aspect of CAF-related miRNAs in the TME is their support in the development of tumor cell drug resistance. Anti-cancer drugs, regardless of their administration route, must diffuse from the bloodstream to individual tumor cells. Generally, the efficacy of these drugs to reach the cancer cells depends on the vascular density of the tumors and the cellular uptake and efflux [91]. However, also the development of chemotherapeutic resistance in tumor cells can be traced to CAF-derived exosomes through a variety of dysregulated miRNAs [92].

One study, investigating whether miRNAs are involved in the reprogramming of normal fibroblasts in breast cancer, identified a signature of miRNA-155, miRNA-31, and miRNA-214, being responsible for the transformation from normal cells to CAFs [93]. Further analysis by this group revealed chemokines as the most highly regulated genes during this process and identified CCL5 as a direct target for the most significantly downregulated miRNA-214. Similar data were found for pancreatic cancer, where Pang et al.

showed that these cancer cells reprogram normal adjacent fibroblasts into CAFs by means of secreted exosomes containing miRNA-155, with the conclusion that the tumor protein p53-inducible nuclear protein (TP53INP) 1 is a target of miRNA-155 in fibroblasts and that a down-regulation of TP53INP1 protein could contribute to the fibroblasts' activation [94]. Further investigations by other groups found miRNA-21 to induce CAF formation via SMAD7. Specifically, miRNA-21 binds to the 3′-UTR of SMAD7 mRNA and inhibits its translation. Normally, SMAD 7 is bound to SMAD 2 and 3, which competitively bind to TGFBR1, and prevents their activation upon TGF-β1 stimulation. Therefore, an overexpression of miRNA-21 or the depletion of SMAD 7 can be critical regulators during the induction of CAF formation [95]. Another study showed that exposure of primary normal human fibroblasts to TGF-β1 resulted in the acquisition of a CAF-like phenotype. This was associated with increased expression of miRNA-145, a miRNA predicted in silico to target multiple components of the TGF-β signaling pathway. Vice versa, overexpression of miRNA-145 blocked TGF-β1-induced myofibroblastic differentiation and reverted CAF toward a normal fibroblast phenotype. Thus, miRNA-145 is a key regulator of the CAF phenotype, acting in a negative feedback loop to reverse acquisition of myofibroblastic traits. This is a key feature of CAFs being associated with poor disease outcome [96]. In prostate cancer, hypoxia-induced miRNA-210 was able to increase the senescence-associated features of normal fibroblasts and converted them into CAF-like cells, able to promote cancer cell EMT, to support angiogenesis, and to recruit endothelial precursor cells and monocytes/macrophages [97]. Another study investigating the impact of cisplatin treatment in esophageal cancer found miRNA-27a/b to contribute to resistance to chemotherapy through miRNA-27a/b-induced transformation of normal fibroblasts into CAFs [98]. Similarly, miRNA-21 as an important activator of CAFs was found to regulate matrix metalloprotease (MMP)-3, MMP-9, platelet-derived growth factor (PDGF), and CCL-6 secretion by these cells, which in turn increased not only the migratory and invasive abilities of pancreatic ductal adenocarcinoma cells (PDAC), but also their drug resistance against gemcitabine [99]. Finally, investigations on gastric cancer revealed that the loss of miRNA-141 induced normal fibroblast to obtain a CAF-like phenotype via an upregulation of signal transducer and activator of transcription (STAT) 4, a direct target of miRNA-141 [100].

The importance of exosomal miRNA transfer from CAFs to cancer cells for tumor growth became obvious by a study investigating the communicative paths between HCC and CAFs [101]. These authors found a significant reduction of miRNA-320 amounts in CAF-derived exosomes. Normally, this miRNA exerts anti-tumor effects by targeting PBX homeobox (PBX) 3 to suppress cancer cell proliferation, migration, and metastasis by suppressing the activation of the mitogen-activated protein kinase (MAPK) pathway, which can induce EMT and upregulate cyclin-dependent kinase 2 (CDK2) and MMP2 expression [101]. In breast cancer cells, three miRNAs (miRNAs -21, -378e, and -143) were increased in exosomes from CAFs as compared with normal fibroblasts, thereby promoting the stemness and EMT phenotype of breast cancer cells via exosomal transfer [102]. Another example is miRNA-148b, which seems to be decreased in CAF-derived exosomes of endometrial cancer. This is an important observation because in vitro and in vivo studies revealed that miRNA-148b normally functions as a tumor suppressor by directly binding to its downstream target gene DNA methyl transferase (DNMT) 1 to suppress EMT and metastasis [103]. Therefore, it was suggested that CAF-mediated endometrial cancer progression is partially related to the loss of miRNA-148b in the exosomes of CAFs [104]. Wang et al. found that miRNA-1228 is increased in CAFs and their secreted exosomes. While investigating osteosarcoma cells, this study demonstrated that CAF-derived exosomal miRNA-1228 is able to promote osteosarcoma invasion and migration by targeting suppressor of cancer cell invasion (SCAI) in the recipient cells [105]. Finally, it was shown that CAF-derived exosomal miRNAs-34a-5p and -3188 play an important

role for proliferation and metastasis in oral squamous cell carcinoma (OSCC) and head and neck cancer (HNC) [106, 107]. Both miRNAs were found to be significantly reduced in CAFs and their exosomes, leading to increased cancer progression. MiRNA-34a-5p leads to an increased expression of its direct target AXL receptor tyrosine kinase (AXL) and the activation of the AKT/glycogen synthase kinase 3 (GSK)-3β/β-catenin signaling pathway, which can induce EMT to promote cancer cells metastasis [106]. MiRNA-3188 can regulate the proliferation and apoptosis of HNC cells by directly targeting B-cell lymphoma 2 (BCL2) in vitro and in vivo [107]. Exosomal transfer was also shown from tumor cells to fibroblasts. One example is the exosome-mediated delivery of miRNA-9 to normal breast fibroblasts. Tumor-secreted miRNA-9 can be transferred via exosomes to recipient normal fibroblasts and this uptake results in enhanced cell motility. Moreover, this miRNA is also secreted by fibroblasts and in turn able to alter tumor cell behavior by modulating the expression of its direct target E-cadherin [108].

Finally, emerging evidence has suggested that deregulated miRNAs play a crucial role in changing the metabolism in CAFs and their surrounding cancer cells. A first study investigating the role of miRNA-186 during CAF formation came to the conclusion that, besides its implication in cell cycle progression, the downregulation of this miRNA led to an increased expression of its direct target glucose transporter (Glut) 1, which is responsible for glucose uptake and lactate production in cells [109]. Another study claiming that CAFs provide metabolites for tumor growth and undergo metabolic reprogramming to support glycolysis came from Zhang et al. [110]. By investigating the TGF-ß1 or PDGF-induced switch of these cells from oxidative phosphorylation to aerobic glycolysis, they identified miRNA-424 as being responsible for the downregulation of isocitrate dehydrogenase (IDH) 3α. Thus, the downregulation of IDH3α results in hypoxia-inducible factor prolyl hydroxylase (PHD) 2 inhibition and HIF-1α protein stabilization, which in turn promotes glycolysis by increasing the uptake of glucose [110]. Representative miRNAs involved in the transformation and activation of CAFs, known to be key regulators of cancer cells, are demonstrated in Fig. 1.2.

1.4 Role of miRNAs on Pericyte Function in the TME

Vascularization is a key process in the pathophysiology of cancer and strongly mediated by various components of the TME. Vasculature development and maintenance are based on nontransformed cell, essentially endothelial cells and pericytes. Both cell types represent the major cellular components of tumor blood vessels within the TME, and angiogenesis during tumor development is inseparably connected to the function of these cells. Pericytes are specialized mesenchymal cells present at intervals along the wall of capillaries in juxtaposition to endothelial cells, where they share and co-produce a basement membrane with them [111–113]. This close proximity enables pericytes to directly stimulate endothelial cell proliferation by the secretion of growth factors and to modulate the surrounding ECM to guide endothelial cell migration [114, 115]. In consequence, several soluble mediators and surface receptors are found to facilitate remodeling through this endothelial–pericyte interaction. Among them, TGF-β, platelet-derived growth factor (PDGF-B), platelet-derived growth factor receptor (PDGFR)-β, angiopoietin 1, angiopoietin 2, angiopoietin receptor-2, and VEGF are the best-studied factors [111]. Moreover, pericellular proteases, for example, membrane-type (MT-) MMPs, serine proteases, cysteine cathepsins, and membrane-bound aminopeptidases, play an important role to support neovascularization by activating or modifying angiogenetic growth factors, and by degrading the endothelial and interstitial matrix [116]. Additionally, cancer stem cells derived from solid tumors can give rise to vascular endothelial cells and were found to function as pericyte progenitors activated through EMT-promoting transcription factors like Twist, Snail, and Zinc finger E-box-binding homeobox (Zeb), activating Wnt,

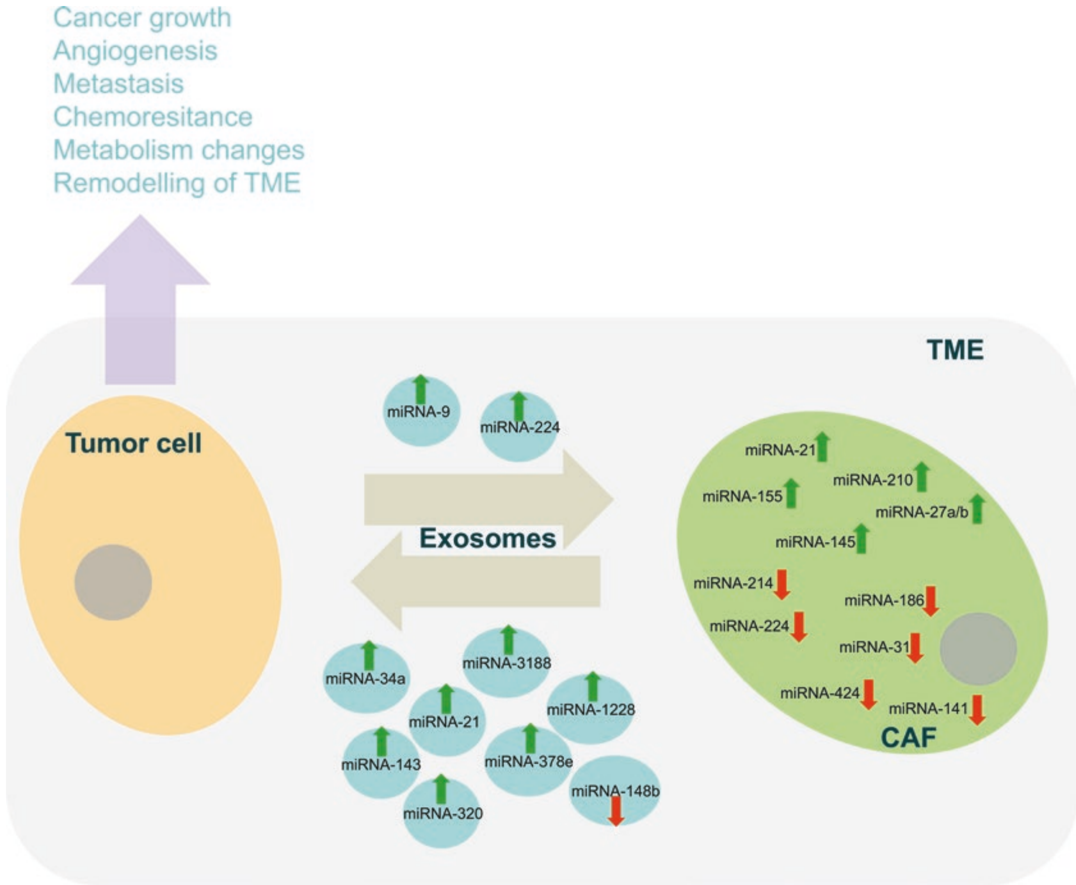

Fig. 1.2 MiRNAs involved in the transformation and activation of CAFs. Tumor-promoting CAFs and miRNAs are key regulators of cancer cells through the regulation of cancer-promoting events, transformation of normal fibroblast into CAFs, and a direct crosstalk through exosomal transfer

NOTCH, TGF-β and nuclear factor-kappa B (NF-κB) pathways, or hypoxia [112, 117].

First reports of a pericyte-specific expression of miRNAs came from a study by Larson et al. [118]. Here, the authors identified miRNA-145 as being selectively expressed in microvessel pericytes, and validated the E26 transformation-specific (ETS) transcription factor Friend leukemia virus integration 1 (Fli1) as a direct target for this miRNA. Moreover, the expression was correlated with increased migration of microvascular cells in response to growth factors [118]. Interesting results for a potential role of miRNAs expressed by pericytes in the TME under hypoxia came from experiments on cultured primary central nervous system pericytes. After exposure to hypoxic stress, several miRNAs such as miRNA-322, miRNA-345, miRNA-345-5p, miRNA-145, miRNA-150, miRNA-140, miRNA-126, miRNA-376-3p, and miRNA-222 were induced by this treatment, suggesting a similar expression pattern of TME-associated pericytes under this condition [119]. Among those investigated miRNAs, miRNA-345-5p showed the most prominent increased expression, leading to the possible regulation of cell cycle progression through targeting cyclin-dependent kinase inhibitor 1 (CKI1) or the downregulation of an anti-apoptotic protein target BcL12-associated athanogene 3 (BAG3), a protein involved in the suppression of cancer cell proliferation and invasiveness in vitro [120, 121]. Similarly, pericyte expression of Let-7d in response to low oxygen conditions was observed during cell differentia-

tion, leading to angiogenesis [122]. In a more comprehensive approach, a systematic study investigated the effect of hypoxia on the expression of pericyte-derived miRNAs and identified miRNA-532-5p to be one of the most differentially modulated among 379 investigated miRNAs. Further investigations revealed the leucine zipper transcription BTB domain and CNC homolog 1 (BACH1) as a direct target, leading to transcriptional regulation of angiopoietin-1 promoter activity and protein expression, as well as enhanced microvascular maturation [123].

Emerging lines of evidence for extracellular vesicle-mediated miRNA exchange between endothelial cells and pericytes came from a study revealing that inflammatory signals are exchanged between these cell populations, leading to increased VEGF-B expression at both the transcriptional and protein levels [124]. Combined investigations on differentially expressed mRNA profiles between samples from glioblastoma microvasculature and glioblastoma tumor cells and corresponding miRNA analysis identified various miRNAs targeting PDGFRB and EGFR during microvascular proliferation [125]. Specifically, miRNA-193b-3p, miRNA-518b, miRNA-520f-3p, and miRNA-506-5p targeting PDGFRB were downregulated in microvascular proliferation, whereas increased expression of miRNA-133b, miRNA-30b-3p, miRNA-145-5p, and miRNA-146a-5p targeting EGFR was observed [125].

1.5 MiRNAs as Modulators Between Infiltrated Immune Cells and Tumor Cells

1.5.1 Cluster of Differentiation (CD)4^{+}, CD8^{+}, and T-reg

As mentioned initially, different types of infiltrated immune cells are pivotal components of the heterogeneous cell population constituting the TME. Depending on the tumor type, these types of cells can be found in variable proportions and represent different phenotypes with either pro- or anti-inflammatory properties [9]. Among them, regulatory T-regs are known to be a subset of immunosuppressive infiltrated lymphocytes involved in the immune escape. Together with TAMs, they produce a favorable environment for cancer cells. On the other hand, T-helper cells (Th), together with CD8+ T cells, NK and DCs, are crucial for tumor suppression [126]. Th cells can be further phenotypically characterized into Th1, Th2, and other Th subpopulations based on distinct profiles of cytokines, transcription factor, and homing receptor expression [127]. Among them, Th1 cells are the main CD4^{+} T-cell population involved in the response against tumors. In contrast to them, T-regs avoid autoimmune reactions and stop the effector response against exogenous antigens if required [127].

First hints for miRNAs and their biogenesis as regulators in the regulation of T-cell functions came from Dicer-deficient CD4^{+} T cells. Here, the authors conclusively demonstrate that Dicer regulates diverse aspects of T-cell biology, including basic cellular processes such as proliferation and survival, as well as cell lineage decisions and cytokine production during Th differentiation [128]. Another study found that the loss of Drosha is responsible for spontaneous T-cell activation, inflammatory disease, and premature lethality. Moreover, both Drosha and Dicer were shown to be critical for the induction of forkhead box P3 (FOXP3) and related functions of the T-reg lineage, since the transcription factor FOXP3 is essential for the differentiation and function of regulatory T-regs. Additionally, the transcription factor GATA-3 is induced in T-reg cells under inflammatory conditions and stabilizes FOXP3 to avoid the differentiation of T-reg cells into inflammatory-like T cells [129]. Another study found that the GATA-binding protein (GATA) 3-inducible miRNA-125a-5p can reverse the suppressive effect of T-regs by targeting IL-6R and STAT3, as direct inducers of Foxp3 [130]. Similarly, Yang et al. found that T-reg cell activation after IL-6 stimulation is additionally controlled by miRNA-17 through its direct target eosinophilia (Eos), a pivotal co-regulator of FOXP3 [131].

Other reports indicated that expression of miRNA-155 and miRNA-146 is induced by pro-inflammatory stimuli such as IL-1, TNF-α, and Toll-like receptors (TLRs) [132]. Moreover, interferon-γ receptor α-chain (IFN- γ Rα) was discovered as a second miRNA-155 target in T cells, suggesting that miRNA-155 contributes to Th1 differentiation in CD4+ T cells by inhibiting IFN- γ signaling [133]. Similarly, it was shown that besides miRNA-155, also miRNA-147, miRNA-146a, and miRNA-132 can be activated by paclitaxel treatment, leading to the expression of the Th1-specific cytokines IFN- γ and IL-12 [134]. Interestingly, another study conclusively demonstrated that miRNA-146a, as one of the miRNAs prevalently expressed in T-reg cells, is critical for their suppressor function due to augmented expression and activation of STAT1, a direct target of this miRNA [135]. Moreover, it became obvious that the TME induces a down-regulation of miRNA-17-92 cluster expressing T cells, thereby preferentially diminishing the persistence of tumor-infiltrated Th1 cells [136]. Further investigations revealed that miRNA-17 and miRNA-19b are the key players controlling Th1 responses by targeting PTEN as well as cAMP-responsive element binding protein (CREB) 1, and that the loss of the miRNA-17-92 cluster in $CD4^+$ T cells results in tumor evasion [137]. Using miRNA arrays, another group found that overexpression of miRNA-568 can inhibit the activation and function of both CD4+ T cells and T-reg cells by targeting nuclear factor of activated T cells (NFAT) 5, and that this prohibition of T-reg-cell differentiation can inhibit the suppressive effect of these cells on effector cells [138].

The observation that tumor-derived exosomal miRNAs modulate T-cell function came from investigations on NPC. For example, NPC cell-derived exosomes impaired T-cell function by inhibiting T-cell proliferation, Th1 differentiation, and by promoting T-reg induction in vitro. Moreover, those exosomes increased the proinflammatory cytokines IL-1β, IL-6, and IL-10, but decreased IFNγ, IL-2, and IL-17 release from $CD4^+$ cells. Further investigations identified miRNA-24-3p, miRNA-891a, miRNA-106a-5p, miRNA-20a-5p, and miRNA-1908 in the exosomes, which were found responsible for a down-regulation of the MARK1 signaling pathway to alter cell proliferation and differentiation [139]. Later, more detailed mechanistic studies revealed FGF11 as a direct target of miRNA-24-3p, this being involved in tumor pathogenesis of NPC by mediating T-cell suppression [140]. Another miRNA mediating immunosuppression in the TME is miRNA-124, which it typically absent in all forms of gliomas. MiRNA-124 targets the STAT3 pathway and reverses the existing glioma cancer stem cell-mediated immunosuppression of T-cell proliferation and induction of Foxp3 expressing T-regs. Moreover, under normal conditions, this mRNA induces effector cell response through upregulation of interleukin 2, IFN-γ, and TNF-α [141].

T-regs in the TME are also found to be recipients for miRNA-214, delivered by cancer cell-secreted exosomes. Such induced T-reg cells are characterized by a reduced expression of PTEN as a direct target of miRNA-214, and secrete higher levels of IL-10 to promote tumor growth [142]. Tumor cells with decreased expression of miRNA-141 have been found in the specific tumor microenvironment that facilitates NSCLC progression. This lack of expression leads to an increased production of CXCL1 and the activation of CXCR2, which in turn recruits T-regs to infiltrate this TME. Since CXCR2 has been demonstrated to be a potent pro-tumorigenic chemokine receptor that directs recruitment of tumor-promoting leukocytes into tissues, the immune escape of tumor cells is supported under these conditions [143]. Finally, it was shown that the downregulation of miRNA-545 promotes T-reg infiltration into the TME of lung cancer, by targeting the chemokine CCL-22 responsible for facilitated T-reg migration and proliferation in lung cancer [144].

MiRNAs can also interfere with the development of cytotoxic T-lymphocytes (CTLs) from activated CD8+ T cells to target the antitumor effect of these cells in the TME. Under normal conditions, these cells are characterized by the upregulation of lytic molecules, such as perforin or granzyme, to perform their response on cancer

cells [145]. Using a genome-wide approach to assess miRNA expression during CTL differentiation, Trifari and colleagues identified miRNA-139, miRNA-342, and miRNA-150 to be involved in this process. They showed that miRNA-139, and to a lesser extent miRNA-342, regulates perforin, whereas miRNA-150 regulates the expression of IL-2 receptor α chain (CD 25). Moreover, IL-2 receptor and inflammatory signals downregulate Dicer expression through a posttranscriptional mechanism, which potentially involves the miRNA lethal (let)-7a-1 [146]. Another study investigated the expression of miRNA-23a in tumor-infiltrating CTLs from lung cancer tissue. Here, the authors found that tumor-derived TGF-ß directly suppresses CTL immune function by elevating miRNA-23a expression, leading to downregulation of granzyme B [147]. Moreover, tumor-derived TGF-β was shown by Yu and colleagues to induce miRNA-491 expression, a negative regulator of CD8+ cells in the TME [148]. Representative illustrations of networks of miRNAs as modulators of tumor infiltrating immune cells, especially via exosomes, are shown in Fig. 1.3.

1.5.2 TAMs

Macrophages are another type of well-characterized tumor-infiltrating immune cells involved in the regulation of TME functions. These myeloid cells belong to the mononuclear phagocytic system (MPS) together with monocytes and DCs. Macrophages ingest and digest degraded dead cells, debris, or foreign material, and are known to be multifunctional antigen-presenting cells. Depending on the ability of such a stimulus to induce inflammatory responses or to antagonize inflammatory responses, macrophages are defined as macrophage type 1 (M1) or M2 polarized cells [149]. Interestingly, tumors are abundantly populated by macrophages. Remarkably, those macrophages during cancer-initiating conditions are indeed immune-activated, while this status is reversed during establishment of the tumor, where they can become even pro-tumoral [150]. Over time, it became obvious, that a specific subpopulation of macrophages carries a pivotal role within the TME. They are part of the host antitumor response and called TAMs, but several studies have shown that many of these TAMs do not protect against the malignant tissue, but rather promote tumor initiation, progression, and metastasis [151]. One reason is the release of inflammatory cytokines leading to a chronic inflammatory environment which is permissive for tumor initiation and progression. Moreover, TAMs can switch from an immune-active state to an immune-suppressive phenotype over time and can polarize into both, M1 and M2, phenotypes depending on the stage of carcinogenesis. Therefore, one of the hallmarks of malignancy is the polarization of TAMs from a pro-immune (M1-like) phenotype to an immune-suppressive (M2-like) phenotype [152]. To make things worse, TAMs critically contribute to the remodeling of the TME through the expression of various proteases and are recruited by hypoxic conditions (see also Sect. 1.2) to induce the angiogenic switch and vascularization of the growing tumor [153].

An initial report that TAM-derived miRNAs can be players in the regulation of cancer invasiveness came from Yang et al. [154]. In the exosomes of Il-4-activated macrophages, the authors identified miRNA-223 as being able to increase invasion of breast cancer cells. In parallel, another study aimed to investigate the role of miRNA-501-3p containing exosomes derived from TAMs in the progression of PDAC. While assessing the function of M2-like TAM recruitment in PDAC tissues, this work found that exosomal-transferred miRNA-501 via TGF-β signaling was associated with promotion of metastasis by targeting TGFBR3 [155]. Moreover, Zhong and Yi found that miRNA-720 downregulated in TAMs from breast carcinomas and M2-polarized macrophages and suggested this miRNA to modulate their function by targeting GATA3, a transcriptional factor that plays an important role in M2 macrophage polarization [156]. Furthermore, miRNA-19a-3p is capable of downregulating the M2 phenotype in M2 macrophages and has an important role in the upregulation of Fra-1

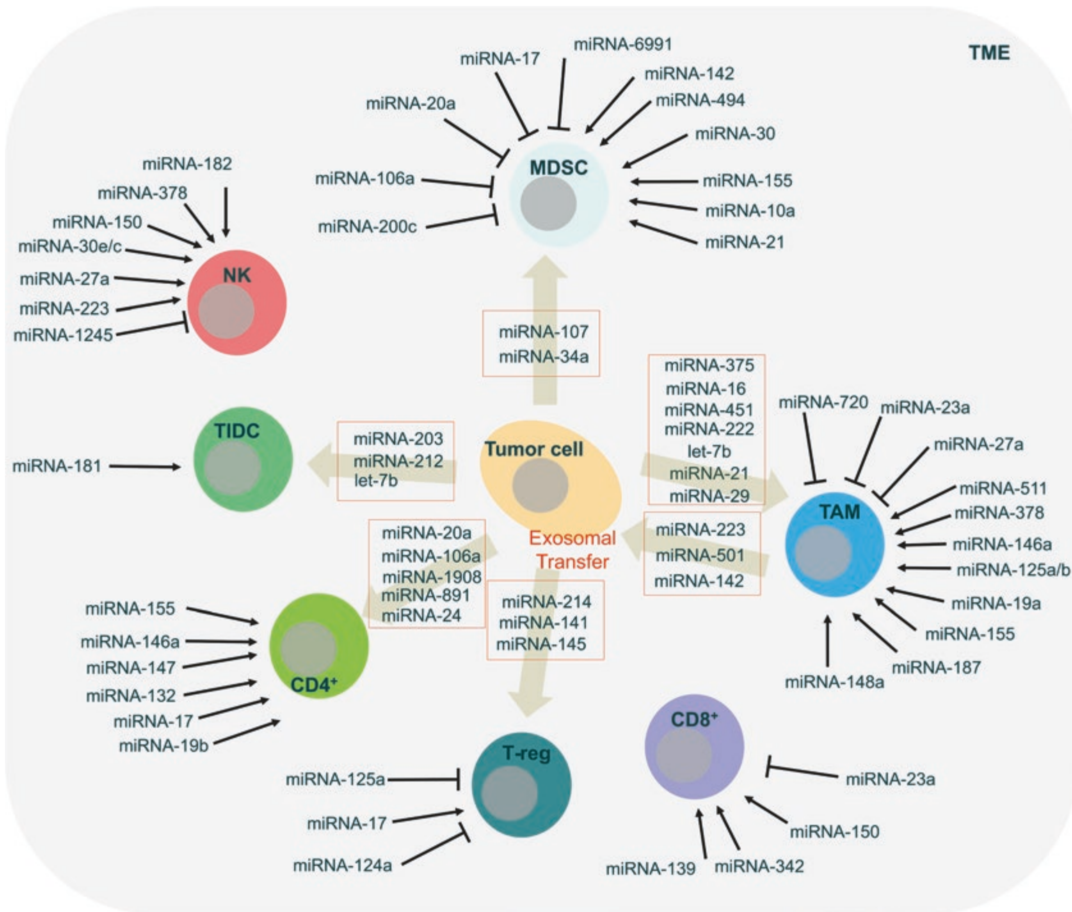

Fig. 1.3 MiRNAs as modulators of tumor-infiltrating immune cells in the TME. MiRNAs expressed in different types of tumor-infiltrated immune and cancer cells regulate immune responses, leading to tumor progression or repression. This network is based, at least in part, on the communication of cancer cells with other tumor-infiltrated cells present in the TME. Among other mechanisms, this can happen via the secretion and transfer of exosomal miRNAs (black arrows indicate an enhancing effect; black arrows with bars indicate an inhibitory effect)

expression and induction of M2 macrophage polarization [157].

Similarly, various miRNA are known to modulate macrophage activation and function within the TME, and a vast majority of them is upregulated via TLR ligands. Examples are miRNA-155, miRNA-125a/b, miRNA-146, miRNA-21 and let-7b, miRNA-187, miRNA-378-3p, and miRNA-511-3p, among others [30, 158–162]. Specifically, Merline and colleagues found that the extracellular matrix proteoglycan decorin controls inflammation and tumor growth through programmed cell death 4 (PDCD4) and miRNA-21 [160]. Decorin not only acts as an exogenous ligand of TLR 2 and 4 to stimulate the production of pro-inflammatory molecules but also prevents the translational repression of PDCD 4 by decreasing the activity of TGF-β1 and the abundance of oncogenic miRNA-21, a translational inhibitor of PDCD4 [163]. Finally, increased PDCD4 leads to a decreased release of the anti-inflammatory cytokine IL-10, rendering the cytokine profile of theses TAMs more pro-inflammatory [160]. Other studies have shown that miRNA-155 regulates inflammatory cytokine production in TAMs via targeting

CCAAT enhancer binding protein (C/EBP)-β, is a critical translational regulator of macrophage programming and activation, and promotes M1 polarization [159, 161, 164]. Another example is the intronic miRNA-511-3p, encoded by the human *mannose receptor C-type (MRC) 1* gene. Activation of this gene during the polarization of pro-tumoral TAM triggers a negative-feedback response initiated by miRNA-155 expression, with the consequence to attenuate this behavior and to inhibit tumor growth [162].

MiRNAs shown experimentally to transfer from macrophages to cancer cells are miRNA-223 and miRNA-142-3p [165, 166]. Zhu et al. observed that hypoxic epithelial ovarian cancer (EOC) cells triggered macrophage recruitment and induced macrophages into a TAM-like phenotype. Moreover, in the context of hypoxic TME, those macrophages contribute to the malignant phenotype of EOC, by secreting exosomal miRNA-223 to promote drug resistance via the PTEN/phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/AKT pathway [165]. Interestingly, an earlier report from Aucher and colleagues showed that the transfer of miRNAs-223 and 142-3p by direct cell contact through gap junctions from macrophage to HCC cells inhibits proliferation and tumor growth, demonstrating the versatile effects of those miRNAs in cancer development [166]. Another report concluded that let-7b expression is responsible for the polarization of prostatic TAMs because downregulation of this miRNA in these cells led to expression changes of inflammatory cytokines, such as IL-12, IL23, IL10, and TNF-α [167].

On the other hand, an enormous amount of work was done to understand the impact of tumor-derived miRNAs in the communication between tumor cells and macrophages. In this context, it became obvious that the activation of macrophages, such as the influence of let-7b on the polarization of TAMs, can be triggered significantly by the tumor cell itself. Thus, it was shown that TLR4 signaling is able to trigger the release of microvesicles by HCC cells, which transfer miRNA let-7b to macrophages in the TME, resulting in the downregulation of IL-6 [168]. Similarly, the delivery of let-7b in a breast cancer mouse model was able to reactivate TAMs by acting as a TLR-7 agonist and suppressing IL-10 production and reversed the suppressive tumor microenvironment to inhibit tumor growth [169]. Furthermore, it was demonstrated that tumor-secreted miRNA-29 and miRNA-21 are able to trigger pro-metastatic and inflammatory responses in macrophages through TLR-8 signaling [170]. In line with these data, cancer-secreted miRNA-222-3p was able to induce polarization of macrophages into the M2 phenotype of TAMs. This was brought about by a direct targeting of suppressor of cytokine signaling (SOCS) 3, which in turn increased the expression and activation of STAT3 to facilitate cancer progression [171]. The immunosuppressive function of miRNA-451, together with miRNA-21, delivered in microvesicles from primary human glioblastoma (GBM) to macrophages was shown by a study from van der Vos and colleagues [172]. Here, the authors demonstrated that exposure of macrophages to these microvesicles decreased c-Myc, a transcription factor driving the expression of many genes connected to various biological processes, like proliferation or apoptosis [172]. Another example is miRNA-16, since investigations on epigallocatechin gallate (EGCG)-treated murine breast cancer cells revealed that they upregulate miRNA-16, which can be transferred to TAMs via exosomes to inhibit TAM infiltration and M2 polarization [173]. Apoptotic tumor cell-derived exosomal miRNA-375 uptake by macrophages via the TGF-β receptor in the TME of breast cancer was shown to be required for their infiltration. Here, miRNA-375 directly targets tensin (TNS) 3 and paxilin (PXN) to enhance the migration capacity of macrophages [174].

Moreover, multiple cytokines expressed in the TME, such as IL-4, IL-6, IFN-β, or CCL2, are known to modulate miRNA expression and activity of macrophages via paracrine signaling [175]. For example, an investigation on the function of the miRNA-23a/27a/24-2 cluster in breast cancer revealed that these miRNAs are positive regulators of pro-inflammatory responses induced by M1 stimulation in macrophages via IL-4, and a negative regulator of M2 polarization. Further

analysis showed that miRNA-23a is responsible for promoting M1 polarization by targeting zinc finger protein A20 (A20), a TNFAIP3 protein that negatively regulates NF-κB-dependent gene expression, which acts as an important negative regulator of immune responses. Additionally, miRNA-23a and miRNA-27a were identified to inhibit M2 polarization by targeting JAK1/STAT6 and IRF4/PPAR-gamma [176]. Another study identified miRNA-195-5p to be deregulated in primary tumors of colorectal cancer (CRC) patients. This miRNA, by targeting notch receptor (NOTCH) 2, is able to suppress GATA3-mediated IL-4 secretion in CRC cells, leading to the inhibition of M2-like TAM polarization [177]. Similarly, Huang and colleagues found that miRNA-146a is responsible to suppress the pro-inflammatory M1 macrophage switch. Additionally, this mRNA is able to promote M2 macrophage polarization through targeting NOTCH1 and to increase the expression of poly (ADP-ribose) polymerase (PARP) γ, which promotes M2 macrophage activation after IL-4 stimulation [178]. Another miRNA involved in TME-associated macrophage polarization is miRNA-148a. In prostate cancer, long non-coding (lnc) RNA colon cancer-associated transcript 1 (CCAT1) expression was found to promote IL-4-stimulated macrophages polarization to M2 and tumor invasion, which was accompanied by the upregulation of miRNA-148a and translational repression of protein kinase C (PKC) ζ [179]. For glioblastoma, TAMs with a phenotype resembling M2-macrophages were associated with tumor progression and the suppression of tumor-specific immunity [180]. In this regard, Taenaka and colleagues have discovered an aryl hydrocarbon receptor (AHR)-dependent transcriptional program to modulate TAM recruitment. Using tumor-conditioned media from glioblastoma cells on glioblastoma-infiltrating TAMs, they identified IFN-β and IL6 to be responsible for the induction of aryl hydrocarbon receptor (AHR) expression in these macrophages. These cytokines are known activators of STAT1 and STAT3 signaling, increasing AHR expression and migration of TAMs toward a CCL2 gradient in vitro. Moreover, decreased miRNA-29b was found to regulate AHR expression under these conditions [181]. In this context, another study evaluating the influence of IL-6 on the signaling between TAMs and CRC cells identified this cytokine to be required for TAM-induced EMT of CRC cells. TAMs enhance CRC cell migration, invasion, and metastasis by regulating the Janus kinase (JAK) 2/STAT3/miRNA-506-3p/FOXQ1 axis, which in turn leads to the production of CCL2 which promotes macrophage recruitment [182]. A representative illustration of the network of miRNAs as modulators of TAMs and tumor cells, especially via exosomes, is shown in Fig. 1.3.

1.5.3 MDSCs

MDSCs were first characterized in tumor-bearing mice or in patients with cancer and represent a heterogeneous population of cells that share their myeloid origin, immature state, and ability to potently suppress T-cell responses [183]. These bone marrow-derived cells are directly involved in the suppression of the immune system during cancer development, and became the objective of numerous studies to explain the potential link between inflammation and tumor progression in the TME [184]. The development of MDSCs is driven by a complex network of signals leading either to accumulation of immature myeloid cells or to their pathological activation [185]. These factors are either produced by tumor cells and promote the expansion of MDSCs through stimulation of myelopoiesis, while inhibiting their differentiation in mature myeloid cells, or by activated T cells and the tumor stroma leading to a direct activation of MDSCs. Common chemokines involved in the migration of MDSCs to tumors are CCL2, CCL5, CCL7, C-X-C motif chemokine (CXCL) 8, and CXCL12 [186, 187]. Factors that induce expansion and activation of these cells can include cytochrome c oxidase subunit (COX) 2, prostaglandins, supercoiling factor (SCF), macrophage-colony-stimulating factor (M-CSF), IL-6, IL-4, IL-13, TGF-γ, IFN-γ, or VEGF activating several signaling pathways in MDSCs that involve STATs and NF-κB [188].

A study aiming to investigate the molecular network regulating the accumulation and function of tumor-expanded MDSCs found that miRNA-494 is a key factor in regulating these processes by targeting PTEN and the activation of the AKT pathway. Moreover, TGF-β was identified to be the main tumor-derived factor responsible for the upregulation of miRNA-494 in MDSCs [189]. Rong et al. demonstrated that the secretion of prostaglandin (PG) E2 can stimulate an expansion of MDSCs by increasing the expression of miRNA-10a. Moreover, AMP-activated protein kinase (AMPK) was identified as a downstream factor in response to miRNA-10 upregulation, and those activated MDSCs were able to inhibit $CD4^+$ T-cell activity [190]. Another group investigated the role of miRNA-6991-3p in the function of MDSCs and identified this miRNA as a suppressor during activation and expansion through translational repression of galectin (LGAL) S9, a β-galactoside-binding protein implicated in modulating cell–cell and cell–matrix interactions, leading to diminished STAT3 activation [191]. Similarly, Xu and colleagues found miRNA-30 expression to be elevated in bursa of Fabricius (B)-cell lymphoma-derived MDSCs and identified SOCS3 as a direct target. They further were able to show that miRNA-30 facilitates tumor growth by increasing differentiation and the immunosuppressive capacity of those cells [192]. Furthermore, it was found that miRNA-9 is able to promote the differentiation of MDSCs by targeting the myeloid differentiation protein runt-related transcription factor (RUNX) 1 [193]. A negative regulation on the differentiation and activity of TME-associated MDSCs was reported by Fontana et al., who investigated the miRNA-17-5p-92 cluster. They found that, during growth and differentiation of monocytic precursors, RUNX1 gets upregulated and activates the expression of the CSFR, this being due to the 3´-UTR of RUNX1 being a direct target for miRNAs 17-5p, 20a, and 106a [194]. As mentioned above, increased concentrations of chemokines in the local microenvironment may promote the infiltration of MDSCs into the tumors, and the infiltrated MDSCs themselves are an important source of these chemokines. In this context, miRNA-155 was identified as a regulator of CXCL1, CXCL2, and CXCL8 expression in MDSCs, contributing to the enhanced recruitment of other MDSCs into the TME by targeting HIF-1α as a potent inducer of these chemokine families [195].

A direct influence of tumor cells on the development of myeloid precursor cells into MDSCs was shown by a study investigating the interplay between Twist and miRNA-34a during this process. The authors found that tumor-derived IL-10 and TGF-β are responsible for the differentiation of MDSCs and conclusively demonstrated that reduced miRNA-34a expression in the tumor cells is responsible for this effect [196]. Conclusive data on gastric-cancer secreted exosomes which are able to deliver miRNA-107 to host MDSCs came from a study by Ren et al. [197]. In this work, they demonstrated that, after miRNA-107, the uptake of the expansion and activity of MDSCs are promoted by the miRNA targeting Dicer1 and PTEN. Moreover, miRNA-34 has been described to act as a critical negative regulator of MDSC expansion, this being brought about by the release of membrane-bound extracellular vesicles from tumor cells [198]. MiRNA-34a expression was also found to alter the expression of the N-Myc proto-oncogene, inhibiting MDSC apoptosis [199]. MiRNA also seems to be involved in the apoptosis of MDSCs. For example, purinergic receptor p2x, ligand-gated ion channel (p2rx) 7, T-cell-restricted intracellular antigen-1 (Tia1), and pleckstrin homology domain-containing protein, family F, member (plekhf) 1 were predicted as potential targets of miRNA-34a [200]. This is an interesting observation since it was shown that adenosine triphosphate (ATP) release into the TME by cancer cells or by infiltrating inflammatory cells in response to cell damage, hypoxia, mechanical stress, or stimulation by cytokines leads to the activation of P2X receptors [201]. Those extracellular ATP-gated ion channels trigger the inflammatory cascade and their downregulation leads to reduced immune and inflammatory responses of tumor-infiltrated immune cells [202]. Another example for the notion that the

specific conditions of the TME directly affect MDSCs came from a study which investigated the hypoxia-inducible expression of miRNA-10a and miRNA-21 [203]. Here, an upregulation of these miRNAs led to an increase of MDSC expansion and activation by targeting retinoic acid-related orphan receptor α (RORA) and PTEN. RORA differentially regulates inflammatory cytokine production in both innate and adaptive immune responses, for example, as a negative regulator of IFN-γ [204].

The important regulatory roles of miRNAs in tumor MDSCs becomes obvious also in several studies focusing on their direct effects on signaling pathways crucial for the regulation of these in TME-associated cells. In this context, it was found that miRNA-200c expressed in myeloid cells can regulate the suppressive function and differentiation of MDSCs by targeting PTEN and ZFPM2 zinc finger protein (FOG2), and that this miRNA expression can be upregulated by the tumor-associated factor granulocyte-macrophage (GM)-CSF [205]. After induction of miRNA-200c in TME-associated MDSCs, the downregulation of FOG2 and PTEN promotes the activation of PI3K/AKT and mediates the phosphorylation of STAT3. Another interesting observation is the downregulation of miRNA-142-3p in tumor-associated MDSCs during tumor-induced myelopoesis [206]. In order to define the molecular mechanisms, by which miRNA-142-3p controls this differentiation, the gene for the transmembrane chain common to all the receptors for the IL-6 cytokine family was identified as a direct target regulated by this miRNA. Beside this alteration of IL-6 signaling, miRNA-142-3p directly targets gp130 and modulates downstream STAT3 signaling mediated by C7/EBP-β [206]. Moreover, miRNA-155 and miRNA-21 have been demonstrated to have a synergistic effect on STAT3 activity and MDSC expansion by targeting Src homology 2-containing inositol-5′-phosphatase 1 (SHIP) 1 and PTEN [207]. A representative illustration of the network of miRNAs as modulators of MDSCs and tumor cells, inclusively via exosomes, is shown in Fig. 1.3.

1.6 Tumor-Infiltrating Dendritic Cells (TIDCs) and NK Cells

TIDCs are important components in the TME that orchestrate tumor immunosuppression and mediate cancer development. They express a broad range of TLRs and cytokines and process antigen material for presentation on the cell surface through MHC II to $CD4^+$ and $CD8^+$ T cells [208]. Besides that, TIDCs can also interact with NK cells and B cells to bridge the innate and the adaptive immune system [209, 210]. NK cells comprise up to 15% of all circulating lymphocytes and are capable of infiltrating most cancer tissues following activation by cytokines. Similar to other tumor-infiltrating immune cells, they are susceptible for IL-12, IL-15, IL-2 or IFN-α, and β [211]. NK cells can rapidly respond to the presence of tumor cells and initiate an antitumor immune response by producing effector molecules, such as IFN-gamma, perforin and granzyme. These effector molecules synergize to mediate apoptosis of target cells through a mechanism where granzymes diffuse through perforin pores on the plasma membrane of the target cell [212]. Even though a significant correlation between high intratumoral levels of NK cells or DCs and increased patient survival has been shown in several types of cancer, the specific conditions of the TME and tumor-derived factors can activate different mechanisms to impair this anti-tumor immunity [213–215].

One characteristic of tumor-derived soluble factors in the TME is their role to disrupt DC differentiation and their ability to activate immune responses. These factors, like cytokines and growth factors, interfere with the regular function of DCs by activating several intracellular pathways, such as MAPK, JAK/STAT, and NF-κB signaling [216]. In this context, Liang and colleagues found that miRNA-22 inhibits the translation of p38 through binding to the 3´-UTR of its mRNA [217]. As one of the most important members of the MAPK family, protein 38 kinase (p38) signaling impacts on tumorigenesis by activating inflammation-associated cytokines, such as IL-6. In consequence, the authors have shown that miRNA-22 regulates the expression of IL-6

by targeting MAPK signaling via p38 [217]. MiRNA-155, which was shown to play multiple roles for tumor-infiltrating immune cells of the TME, can also regulate TIDCs by mediating the silencing of proto-oncogene c-Fos expression, leading to an increase of IFN-β and IL-12 cytokine production [218].

Data demonstrating that tumor-derived miRNAs can regulate the process of cross-presentation between DCs and effector T cells came from investigations about miRNA-203. In humans, miRNA-203 is frequently overexpressed in solid tumors and often found as cargo in tumor-derived exosomes [219]. Moreover, exosomal miRNA-203 acts as a regulator to control the expression of TLR4 and the production of cytokines such as TNF-α and IL-12, contributing to the dysfunction of DCs [219]. Similarly, it was shown that miRNA-212-3p, via exosomal transfer, is able to target regulatory factor X-associated protein (RFXAP), the key transcription factor for the MHC II gene expression in DCs [220]. An independent study to assess the effects of targeted delivery of let-7b to TIDCs came to the conclusion that this miRNA is able to restore TIDC immunological function, possibly by modulating the content of IL-10, IL-12p70, MMP-9, and VEGF within the TME [169].

A general role of miRNAs in the regulation of NK cell activation, survival, and function has been shown using conditional deletion of Dicer or DGCR8, since the induced ablation of the miRNA biogenesis pathway increased apoptosis of NK cells [221]. The link between microRNAs and NK cell development came from a study showing that miRNA-181 directly regulates the developmental process by regulating NOTCH signaling [222]. Next-generation sequencing of NK-cell transcriptomes revealed that miRNA-223 specifically targets the 3´-UTR of granzyme B [223]. Similarly, miRNA-27a-5p, miRNA-30e, miRNA-150, and miRNA-378 were found to regulate perforin and/or granzyme B to impair the antitumor potential of NK cells in the TME [224–226]. The most intensively investigated miRNA in NK cells within the TME is miRNA-155. This miRNA is required for the normal function of NK cells and expressed during activation of these cells [227]. Thus, it was shown that miRNA-155 plays a central role in NK cell IFN-γ production, by regulating the expression of SHIP1 as a regulator of the PIK3 pathway [225, 228–230]. Moreover, Donatelli and colleagues observed that TGF-ß treated NK cells exhibit reduced tumor cytolysis and abrogated perforein polarization at the interface between antigen-presenting cells and NK cells. Further investigations revealed that TGF-β-induced miRNA-183 expression leads to depleted DNAX-activating protein 12 (DAP12), which is critical for surface NK receptor stabilization and downstream signal transduction [231]. Also, tumor-derived macrovesicles are key mediators between cancer cells and NK cells in the TME. Moreover, this process can explain how hypoxic stress impairs the antitumor immune response by the transfer of suppressive signals to immune cells, because miRNA profiling revealed the presence of high levels of miRNA-23a in hypoxic tumor-derived macrovesicles. Finally, this miRNA has also been shown to directly targeting lysosomal-associated membrane protein (LAMP) 1 after uptake into NK cells [232].

Additionally, NK cells are characterized by several inhibitory and activating receptors. For example, NK group 2 member (NKG2) D is found on NK cells to trigger cytotoxicity against tumor cells, whereas NKG2A is expressed at the cell surface as a heterodimer with CD94. Binding to its cognate ligand inhibits NK-cell effector functions [233, 234]. A study focused on the potential interaction between miRNAs and the 3′-UTR of the *NKG2D* gene, coding for killer cell lectin-like receptor K (Klrk) 1, identified miRNA-1245 as a negative regulator of Klrk 1 in NK cells. Furthermore, it was found that miRNA-1245 expression was inducible by TGF-ß1 by post-transcriptional processing in NK cells. More interestingly, IL-15, which is a potent inducer of *NKG2D* expression, decreased the expression of mature miRNA-1245 significantly [235]. Another group characterized the impact of miRNA-182 on *NKG2A* and *NKG2D* to investigate the role of miRNAs in the activation and cytotoxic function of NK cells from HCC patients. They found a positive correlation between miRNA-182 and the

expression of both receptors. Since they also observed an increased production of perforin-1 and increased cytotoxicity, the impact of miRNA-182 on NK cells still remained unexplored [236]. In this context, Ma and colleagues found that miRNA-30c could promote the cytotoxicity of NK cells by upregulating the expression of NKG2D and Fas cell surface receptor (Fas) ligand, a member of the tumor necrosis factor family which is expressed on the membrane of activated NK cells, being involved in the apoptosis of target cells. Moreover, LAMP 1, a marker for NK cell cytotoxicity, was upregulated via miRNA-30a [237]. A representative illustration of the network of miRNAs as modulators of TIDCs, NK cells, and tumor cells, inclusive of possible exosomal transfer, is shown in Fig. 1.3.

1.7 Immune Checkpoint – A Therapeutic Approach Using miRNAs as Targets or Tools in the Future

Surgery, conventional chemotherapies, and radiation therapy are still cornerstones of today's cancer treatment. However, to achieve a significant further improvement for patients, personalized strategies preventing progression and metastasis are indispensable. As discussed above, the TME is critical in both the initiation and progression of tumor disease. An identification of therapeutic targets in the TME context could be useful to develop alternative approaches for treatment. However, the complex interplay of different TME-associated cells and their specific molecular and cellular mechanisms render it highly likely that manipulating one TME component will lead to ambiguous roles in the prevention of tumorigenesis, progression, and metastasis.

MiRNAs have gained rapid diagnostic and therapeutic value by providing unique expression profiles in metastasizing tumor cells, tumor-infiltrating and associated cells [73, 238]. The intervention of miRNAs at nearly all biological processes and cancer-related pathways observed within the TME promotes them as attractive potential biomarkers and therapeutic targets or agents [239]. Since miRNAs are proposed to act either as tumor-suppressors or as oncogenic miRNAs, some basic strategies for such therapeutic approaches were developed in the past, including miRNA mimics, anti-miRNA oligonucleotides, or miRNA sponges [240]. MiRNA mimic technologies are based on the gene-silencing effect of small ncRNAs acting like mature endogenous miRNAs. Antagonistic oligonucleotides, also referred to as antagomiRNAs or anti-miRNAs, affect miRNA-related pathways by binding and blocking oncogenic miRNAs. These synthetic small RNA molecules have a complementary sequence to the endogenously expressed mature miRNAs and inhibit their function by direct binding [241]. Analog to naturally expressed circRNAs, miRNA sponges are used to scavenge endogenous miRNA expression. These miRNA sponges are synthetic agonists that contain four to ten seed sequences as binding sites, separated by a few nucleotides each. These constructs act as baits by attracting endogenous miRNAs, preventing their interaction with their native targets [242]. To achieve their impact, either as therapeutic agents or as targets of therapeutic inhibition, different types of vehicles exist for their delivery to the targeted cells, including liposomes, polymers, nanoparticles, and viral approaches, besides others [243–245].

One of the most intensively investigated potential approaches to therapeutically influence the TME in the future is the strategy to include miRNAs implicated in the modulation of immune checkpoint molecules [246]. Activation or repression of T-cell functions is tightly controlled by a group of specific immune checkpoint molecules, and intensive investigations revealed the impact of these molecules as important regulators of a patient's immune response in different diseases, including cancer. Therefore, among the different types of cancer immunotherapies, immune checkpoint blockage using several specific antibodies soon became the "state of the art," and the early success in clinical trials led to the approval for patient treatment. However, still numerous cancer patients did not respond and remained uncured by these treatments, leading to the necessity to investigate supplemental or alternative

concepts. For example, ncRNA regulation of immune checkpoints has been studied in cancer for new therapeutic solutions [247].

Under normal physiological conditions, the prevention of autoimmunity is regulated by a balance between co-stimulatory and inhibitory signals or immune checkpoints. Consequently, the immune system can normally distinguish transformed cells from their normal counterparts by a diverse set of antigens. Interestingly, co-stimulatory and inhibitory receptors and ligands that regulate T-cell activation are not necessarily expressed in cancer cells compared to normal tissues. In contrast to that, inhibitory ligands and receptors that regulate T-cell effector functions in tissues are commonly overexpressed on tumor cells or TME-associated cells [248]. Most prominent immune checkpoint molecules are cytotoxic T-lymphocyte-associated antigen 4 (CTLA 4), PD-1, and its ligand PD-L1 [249].

Among them, CTLA-4 was the first checkpoint molecule discovered on the surface of T cells. CTLA-4 prohibits early T-cell activation by counteracting CD28 to prevent TCR signal transduction by binding. PD-1 can be found on several immune cells, including DCs, monocytes, or T- and B cells, upon stimulation [250]. As a member of the B7-CD28 family, it can bind to its ligands PD-L1 and PD-L2, leading to downregulation of pro-inflammatory cytokines and anti-apoptotic signals, as well as reduced T-cell receptor signaling. Both ligands are expressed on antigen-presenting cells, like DCs. The mediation of such inhibitory signals through PD-1 is the cause for T-cell exhaustion, a condition in which T cells lose their effector function(s) and upregulate various inhibitory receptors. Furthermore, such T cells lose their ability to reach a state of naïve T cells termed hyporesponsiveness or quiescence, which is characterized by small cell size, low proliferative rate, and low basal metabolism [251]. PD-L1 itself is stimulated in the TME via inflammatory cell-secreted IFN-gamma, and interferes with immune tolerance in cancer by changing the proliferation of $CD8^{+}$ cytotoxic T cells, the inhibition of cytokine production, and proliferation of tumor infiltrating T cells. TIM3 is also expressed by different types of immune cells, including DCs, NK cells, T cells, and macrophages. It inhibits T helper responses simultaneously by blocking TIM3 and PD-1-enhanced antitumor immunity [247].

Majority of studies investigating the interaction of miRNAs with immune checkpoint molecules to evaluate their potential as novel immunotherapeutic agents have been done at PD-L1 expression in solid tumors. Chen et al. found that miRNA-200 plays a pivotal role in the immunosuppression and metastasis of NSCLC by targeting PD-L1 [252]. Using immunocompetent syngeneic mice, they were able to show that expression of PD-l on tumor cells is targeted by miRNA-200 under the participation of transcription factor ZEB1 and that decreased PD-L1 expression directly correlates with the amount of tumor-infiltrated CD8+ T cells. Therefore, they concluded that exogenous overexpression of miRNA-200 in tumor cells could decrease PD-L1 expression, which in turn supports intratumoral $CD8^{+}$ T-cell immune suppression in the TME of NSCLC [252]. Another potential therapeutic approach for NSCLC has been suggested by a study investigating the pivotal role of miRNA-197 in platinum-based chemotherapy resistance [253]. Using miRNA array technology, this miRNA was identified as being upregulated in a cohort study comparing primary NSCLC tissue with adjacent normal tissue. In a lung cancer xenograft mouse model, miRNA-197 was shown to inhibit cancer progression by targeting CKS1B. Since CKS1B is directly involved in the regulation of PD-L1 expression on tumor cells, exogenous overexpression of miRNA-197 could not only reverse the effect of platinum-based chemotherapy resistance but also support intratumoral immune suppression in the TME [253]. Moreover, PD-L1 could be regulated by the translational inhibition of Cbl proto-oncogene B (Cbl-b) and c-Cbl by miRNA-181 and miRNA-194 [254]. In consequence, STATT3/AKT/ERK signaling responsible for PD-L1 expression was inhibited though this axis. Since patients with a low PD-L1 expression showed a significantly better survival in this study, also these miRNAs can be considered as potential therapeutic agents for NSCLC co-treatment in the future [254].

Shaohua and colleagues were interested to antagonize the upregulation of PD-L1 expression in ovarian cancer and to induce cancer cell-specific T-cell activity [255]. They found that increased miRNA-424 expression is necessary in ovarian cancer cells to suppress its direct target PD-L1, thereby increasing cancer cell response to platinum-based chemotherapy. Furthermore, disruption of the PD-L1/PD-1 pathway through this axis led to the conclusion that this approach might also improve the therapeutic efficacy of chemoresistant tumors associated with PD-L1 overexpression [255]. Other examples of miRNAs that have been found to directly target the 3'UTR of PD-L1 in tumor cells of the TME are miRNAs-138-5p and miRNA-148a-3p in CRC, miRNA-17-5p in melanomas, miRNA-142-5p in pancreatic cancer, miRNA-940 in gastric cancer, miRNA-3609 in breast cancer, or miRNA-34 for malignant gliomas [256–262].

Despite its well-documented role for antibody-based immune checkpoint therapy, the detailed roles of miRNAs in the regulation PD-1 to influence the immune status are, compared to PD-L1, still poorly investigated. One study revealed that miRNA-374b is directly targeting the 3´-UTR of PD-1 in autologous cytokine-induced killer cells (CIK) [263]. These cells, also called T cells with NK phenotype, are activated and expanded peripheral blood mononuclear cells (PBMCs) isolated from patients after priming with CD3 antibodies and a set of cytokines [264]. Using this approach, the authors demonstrated increased IFN-gamma secretion and enhanced adoptive immunotherapy efficacy for liver cancer as a direct consequence of PD-1 downregulation in these cells [263]. The potential role of miRNA-28 as a therapeutic target in cancer immunotherapy was evaluated by a study investigating the effect of miRNAs on PD-1 in melanomas and T-cell exhaustion [265]. Here, the authors conclusively demonstrated that miRNA-28 can convert the exhaustive phenotype of T cells by directly targeting the inhibitory receptor PD-1 on T cells. Moreover, this repression of PD-1 receptor expression recovered the ability of T cells to secrete cytokines, like IL-2 and TNF-α [265]. In this context, miRNA-149-3p was found to be responsible to inhibit CD8+ T-cell exhaustion in breast cancer. Interestingly, miRNA-149-3p alone does not target PD-1 exclusively, but also the other immune checkpoint receptors TIM-3 and BTLA. Similar to the aforementioned miRNA-28, downregulation of PD-1 promotes CD8+ T-cell-mediated immune response and reverses T-cell exhaustion by enhancing the level of T-cell cytokines associated with and mediating T-cell activation, enhancing T-cell proliferation, and reducing T-cell apoptosis and downregulating FOXP1 [266]. Using a syngeneic subcutaneous glioma mouse model, another group investigated the hypothesis that miRNA-138 could regulate T-regs and that administration in vivo could exert potent antitumor immune effects [267]. After demonstrating the direct targeting of the 3′- UTR of PD-1 by miRNA-138 in T-reg, CD4+, and CD8+ T cells, they concluded that the observed therapeutic effect on tumor growth is immune-mediated. This was supported by the observation that intravenous administration of miRNA-138 in a the syngeneic subcutaneous glioma mouse model demonstrated significant reduction of CTLA-4, PD-1 and FOXP3 on CD4+ T cells in the TME of this animals. This provides a rationale for the development of miRNA-138-based treatments, which target multiple mechanisms of tumor-mediated immune suppression, which is further supported by the knowledge that despite effective CTLA-4 blockade, PD-1 becomes upregulated as a compensatory mechanism [267].

The potential of miRNAs to regulate the immune checkpoint is not limited to their direct influence to mediate PD-1 and PD-L1 expression. So far, several miRNAs have been described that potentially regulate their expression via targeting molecules, involved in signaling pathways regulating their expression, which needs to be taken into account to illustrate the multilayer influence of miRNA regulation in checkpoint blockage [249].

One example is IFN-γ-induced pathway. This interferon is primarily produced by cells of the immune system and interacts as a key immunoregulatory protein with nearly every cell type within the TME by inducing inflammatory innate

response and subsequent immune response [268]. IFN-γ signaling is mediated via a cascade of tyrosine phosphorylation events after binding to IFN-gamma receptor (IFN-γR). This binding activates the canonical JAK/STAT signaling pathway, leading to an activation of receptor-associated JAK1 and JAK2 protein-tyrosine kinases, and subsequent tyrosine phosphorylation and activation of primarily STAT1. After translocation to the nucleus, STAT1 binds to conserved IFN-γ activation site (GAS) DNA elements and directly activates the transcription of interferon-stimulated genes (ISGs), such as chemokines or antigen-presenting molecules [269]. As expected, this signaling cascade is also targeted by translational regulation of key components and several miRNAs are found to be involved in this mechanism. For example, a study aimed to investigate the impact of miRNA-24 and miRNA-181 on IFN-gamma secretion and came to the conclusion that these two miRNAs negatively regulate the expression of this interferon by directly binding to target sites within its 3´-UTR [270]. Another miRNA targeting the interferon pathway was described by a study aiming to identify novel interaction regulatory networks based on the crosstalk between miRNAs and the JAK/STAT axis in melanoma [271]. Using a hematopoietic chimera model, this study found an increased expression of miRNA-146a in T cells of melanoma-bearing mice. Further investigations indicated that miRNA-146a is produced in the TME to prevent activation of the STAT1/IFN-γ axis. Furthermore, it became obvious that miRNA-146a plays a central role within the melanoma microenvironment, by affecting melanoma migration, proliferation, cell-cycle activity, and basal metabolic rate through the direct control of IFN γ expression. However, since it was demonstrated that reduced IFN γ expression leads to increased PD-L1 levels on the melanoma cells, this beneficial effect of an antagomiRNA-based treatment still needs to be supplemented with an immune checkpoint blockage using a specific anti-PD-L1 antibody [271]. These data are supplemented by the observation that STAT1 is a direct target of miRNA-15 and miRNA-223 [272].

1.8 Concluding Remarks

Cancer is a multistep systemic disease based on crucial interactions between tumor cells and the specific conditions within the TME. Tumor growth and dissemination strictly depend on an interactive crosstalk between cancer cells and the TME. This complex network is strongly regulated and influenced by the presence of miRNAs able to silence key mRNAs within this network. Thus, miRNAs exert a pivotal influence on the crosstalk between infiltrated immune cells, CAFs, and tumor cells during tumor progression but also support the unique niche for tumor cells to induce angiogenesis and secretion of additional factors to promote tumor cell invasion and metastasis. Thus, the identification of altered miRNA expression in tumor cells, but also infiltrated immune cells, CAFs, further stromal cells under conditions such as hypoxia can shed more light on this complex interplay. This could also potentially lead to overcome treatment failures due to immune-suppressive conditions of the TME, and suggest miRNA-based ideas to reverse therapeutic resistance of cancer cells. In the long term, this pre-clinical knowledge can help to increase the efficacy of even modern personalized therapeutic approaches, such as immune checkpoint inhibitors.

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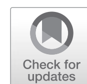

The Impact of Estrogen in the Tumor Microenvironment

2

Ashwin Somasundaram, Natalie J. Rothenberger, and Laura P. Stabile

Abstract

Tumor immune escape is now a hallmark of cancer development, and therapies targeting these pathways have emerged as standard of care. Specifically, immune checkpoint signal blockade offers durable responses and increased overall survival. However, the majority of cancer patients still do not respond to checkpoint blockade immune therapy leading to an unmet need in tumor immunology research. Sex-based differences have been noted in the use of cancer immunotherapy suggesting that sex hormones such as estrogen may play an important role in tumor immune regulation. Estrogen signaling already has a known role in autoimmunity, and the estrogen receptor can be expressed across multiple immune cell populations and effect their regulation. While it has been well established that tumor cells such as ovarian carcinoma, breast carcinoma, and even lung carcinoma can be regulated by estrogen, research into the role of estrogen in the regulation of tumor-associated immune cells is still emerging. In this chapter, we discuss the role of estrogen in the tumor immune microenvironment and the possible immunotherapeutic implications of targeting estrogen in cancer patients.

Keywords

Tumor microenvironment (TME) · 17β-estradiol (E2) · Estrogen receptor α (ERα) or estrogen receptor β (ERβ) · Cancer-associated fibroblasts (CAFs) · Tumor-associated macrophages (TAMs) · Myeloid-derived suppressor cells (MDSCs) · Immune T cells · CD4+ T lymphocytes · CD8+ T lymphocytes · Regulatory T cells (Tregs) · Programmed death-1 (PD1) · Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) · Immune B cells · Natural killer (NK) cells · Dendritic cells (DCs) · Fibroblast growth factor (FGF) · Epidermal growth factor (EGF) · Vascular endothelial growth factor (VEGF)

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2.1 Introduction

The tumor microenvironment (TME) is made up of multiple cell types beyond only tumor cells including immune cells, stromal cells including pericytes, and extracellular molecules all regulating tumor growth. These cells have been well established as mechanisms of resistance and have been targets for cancer therapy [1–4]. While these therapeutic strategies have been promising, de novo, and acquired resistance leading to inevitable tumor progression remains an ongoing problem [5–7]. Therefore, alternative regulatory pathways have become necessary to evaluate for possible avenues for future therapeutic research. Female gender has been suggested in retrospective meta-analyses to be associated with decreased response to checkpoint blockade therapy [8–10]. Given the known role of estrogen and other sex hormones effecting immune responses, these findings warrant the evaluation of estrogen signaling in the TME [11]. Estrogen is a steroid hormone that has many physiological functions associated with reproduction, metabolism, and even immune regulation [12]. The main biological endogenous estrogen, 17β-estradiol (E2), is synthesized from androgens by aromatase (CYP19A1) and binds estrogen receptor α (ERα) or estrogen receptor β (ERβ) to exert its effects through both genomic and non-genomic mechanisms [12–17]. Estrogen has been long established as a driver of malignancy in hormone-sensitive carcinomas such as ovarian, breast, endometrial, lung, colon, and even prostate [18]. The oncogenic function of ER is due to the ability of tumor cells to enable transcriptional upregulation of proliferation and cell-survival genes via growth factors such as insulin growth factor (IGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [19–23]. Therapy targeting these aspects of E2 signaling in cancer has been in use clinically for decades. These therapeutics include selective estrogen receptor modulators or degraders (SERMs or SERDs) and aromatase inhibitors (AIs), and are typically utilized in hormone-positive breast cancer, but their utility is being evaluated in other relevant solid tumors [24]. However, most of the studies have focused primarily on the tumoral signaling of E2, while the remainder of the TME has gone unexplored.

E2 signaling and ER expression are not limited to tumor cells but also found on immune cells where they have distinct functions of immune regulation [25–28]. The link between E2 and autoimmunity has been established since findings of sex disparities in patients with systemic lupus erythematosus (SLE), and multiple, current reviews outline E2 regulation of immune cell function and expansion [29–32]. While the link between E2 and immune regulation has been well characterized and tumor immunology is growing as a field, there is a missing connection between E2 pathways and tumor immunology. This chapter will discuss the current findings in the literature exploring the impact of E2 and tumor immunology, as well as the future therapeutic implications of targeting the E2 pathway in the cancer immunotherapy era.

2.2 E2 Signaling Pathways on Tumor Cells

While ER expression and E2 pathways are canonically associated with tumor cells from hormone-sensitive tumors such as ovarian, breast, and endometrial, there are almost 30 tumor types that are also associated with the E2 pathway [33, 34]. These findings are also associated with changes in outcome for the disease further conveying the importance of understanding this pathway across multiple relevant tumor types. For example, nuclear ERα expression in breast cancer, ovarian cancer, or endometrial cancer is correlated with improved overall survival (OS) compared to cancer patients that are ERα-negative [25, 35–38], while some of the breast cancer patients that were ERα-positive also had increased disease burden. Conversely, cytoplasmic ERα expression in non-small-cell lung cancer (NSCLC) cells is correlated with worse OS [39–41]. Aromatase and ERβ expression in tumor cells are more controversial with studies varying on whether they convey a survival benefit [42–47]. These mixed opinions in the literature are

possibly due to the lack of standardized and clinically validated staining ERβ antibody, as well as the multitude of ERβ splice variants and post-translational modifications [26, 48, 49]. While these findings are consistent with understanding E2 on tumor cells, there is still the need to evaluate the remainder of the TME.

2.3 The E2 Pathway in Tumor-Associated Stromal Cells and Immune Cells

Within the TME, ERs and aromatase are in notable concentrations in stromal and immune cells in addition to neoplastic cells (Table 2.1). A myriad of studies in the past decade have detailed key interactions between neoplastic cells and their recruited stromal cells that are responsible for tumorigenic potentiation (reviewed in [4, 47]). Cellular architecture complicit in this potentiation is heterogeneous between and within tumor cells, but generally includes cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), immune T and B cells, natural killer (NK) cells, and endothelial cells [4]. Transitively, the association of hormonal protein expression in TME stromal and immune cells serves to underlie a potential immunomodulatory role of ER signaling in cancer biology, demonstrated by cell types listed in Table 2.1.

2.3.1 Tumor-Infiltrating Lymphocytes (TIL)

There exists a notable interplay between cancer type and lymphocyte composition of the TME. It is often opposing immune infiltrates within a given primary tumor that promote neoplastic evolution and antitumor immunity [65]. For example, $CD4^{+}$ T-cell polarization has been identified as a mediator of tumor immune surveillance. Specifically, T helper 1 (Th1) T cell responses are associated with tumor suppression while T helper 2 (Th2) exhibit tumor activation via IFNγ and IL-12 upregulation and IL-4 expression, respec-

Table 2.1 Estrogen receptor (ER) and aromatase expression in stromal and immune cells in the tumor microenvironment

TME cell type	Cancer type	Human expression	Murine expression	Method of evaluation	Reference
Stromal	Breast	Aromatase	ERα	PCR, IHC	[50, 51]
	Melanoma		ERα	IHC	[51]
	Lung		ERα	IHC	[51]
	Endometrial	Aromatase		IHC	[52]
CAF	Breast	ERα		PCR	[53]
	Prostate	ERα, ERβ		IHC	[54, 55]
	Endometrial	ERα, ERβ		PCR	[56]
	Ovarian	ERα		IHC	[57]
TAM	Ovarian	ERα, ERβ		IF,IHC	[58]
	Breast	Aromatase		IHC, PCR	[59]
	Lung	Aromatase	Aromatase	IHC	[17, 60]
MDSC	Ovarian	ERα	ERα	PCR, Western	[57]
NK cells	Breast	ERα, ERβ		IHC	[61]
Effector CD4+/CD8+ T cells	Breast Nonmalignant	ERα, ERβ		IHC	[27, 62]
Tregs	Cervical	ERα		IHC	[63]

Table adapted from [64]
Studies were identified by PubMed searches using keywords: ERα, ERβ, aromatase, stromal, CAF, TAM, MDSC, expression, cancer. *CAF* cancer-associated fibroblast, *TAM* tumor-associated macrophage, *MDSC* myeloid-derived suppressor cell, *IHC* immunohistochemistry, *PCR* polymerase chain reaction, *IF* immunofluorescence, *Western*: Western blotting analysis

tively [66, 67]. Interestingly, several murine and human studies have reported an induction of Th2 response and IL-4 production in settings of elevated E2 [29, 32]. Further support of ER's role in tumorigenesis was illuminated by a recent in silico study showing an increase in Th1 T cells, B cells, and cytotoxic T lymphocytes (CTLs) in ER-negative breast tumors relative to ER-positive breast tumors [68]. This study additionally saw an inverse correlation between ER activity and immune infiltration of these cell types in breast cancer tissues. The inverse correlation observed affirmed previous reports that increased TIL, specifically $CD8^+$ T cells, in ER-negative tumors correlated with improved OS [68, 69]. Additionally, post hoc analysis in ER-positive breast cancer patients treated with letrozole showed increased infiltration of B and Th1 cells both at the initiation and at the end of treatments [68].

2.3.1.1 Cytotoxic T Cells and Natural Killer Cells

Granule-mediated exocytosis of serine proteases, such as granzyme B, is a major pathway CTLs and NKCs initiate caspase-dependent apoptosis to eliminate pathogenic and tumor cells [70, 71]. Jiang et al. cultured ERα-expressing human liver carcinoma cells with E2 resulting in upregulated expression of the granzyme B inhibitor, proteinase inhibitor-9 (PI-9). This upregulation protected the tumor cells against granule-mediated exocytosis by these cells per DNA fragmentation assays [72]. A similar study illustrated E2-induced PI-9 expression was also observed in ERα-positive MCF7 breast cancer cells with the same protection, while PI-9 knockdown blocked E2's protective effect [73]. Cumulatively, these studies suggest a component of E2 immunosuppression is via inhibition of NK- and CTL-mediated tumor cell elimination.

2.3.1.2 Regulatory T Cells

T cell activation and effector differentiation are integral to the adaptive immune response. $FoxP3^-$ expressing Tregs subdue neoplastic activity, as well as responder T cell expansion, through secretion of immunosuppressive cytokines [74]. Administration of physiologic doses of E2 to immunocompetent, ovariectomized mice has been observed to expand $CD4^+CD25^+$ Treg concentration, as well as Foxp3 expression in various tissue types [75]. Furthermore, fluorescence-activated cell sorting (FACs) assays revealed acquisition of CD25 in E2-incubated ERα-expressing $CD4^+CD25^-$ cells [75]. These transformed $CD4^+CD25^+$ T cells then exhibited an immunosuppressive Treg phenotype in vitro that significantly downregulated T cell concentration [75–78]. Additional studies have reported E2-stimulated Foxp3 expression in murine Tregs, expression of which is vital to Treg functionality. High $FoxP3^+$ Tregs in the TME is a negative prognostic indicator in a variety of cancers. For example, early-stage NSCLC with nuclear ERα expression has a relatively higher risk of both recurrence and $FoxP3^+$ lymphocyte infiltrate [79]. Furthermore, a recent meta-analysis reported $FoxP3^+$ Treg infiltration correlated negatively with OS in ER-positive breast cancer patients and positively in ER-negative patients [80]. Conversely, studies of ERα-positive breast tumors treated with letrozole in vivo demonstrated a resulting reduction of $FoxP3^+$ Tregs [81].

E2 appears to suppress Treg expression in both physiologic and ERα/ERβ knockout mice, with the former group having increased expression of programmed-death 1 (PD-1) and the latter having decreased PD-1 expression [82]. E2 treatment of ERα-positive endometrial and breast cancer cells also stimulates in vitro expression of the PD-1 ligand (PD-L1) via activation of PI3K signaling [83]. $PD\text{-}L1^+$ tumor cells exhaust $PD\text{-}1^+$ cytotoxic T lymphocytes (CTLs) through this protein interaction, resulting in tumor immune evasion [84]. Given E2's upregulation of both PD-1 and PD-L1, the hormone appears to have an important influence on the pathway and its role in the TME.

2.3.2 Stromal Cells

Tumor evolution is heavily dependent on malignant tissue as well as recruited stromal cells that

interact between and within the TME. Via an in vivo murine model, ERα expression in stromal cells was observed within the context of tumor-cell-independent ER signaling in the TME. E2 interactions with stromal ERα has also been seen to accelerate neoplastic growth and blood vessel density in ovariectomized, syngeneic mice transplanted with ER-negative melanoma, breast, or lung cancer cells [51]. The same study found this E2-stimulated tumor growth demonstrated a relative increase in immunocompromised mice, reflecting closer association with E2 modulation of innate immunity [51]. Aromatase expression appears to also modulate the TME in certain neoplasms. Perineoplastic endometrial stromal cells' expression of aromatase also correlates with more advanced disease and, transitively, worse OS [52, 85]. Similarly, perineoplastic breast adipocytes' expression of aromatase appears to be complicit in tumorigenesis in obese patients via inflammation and modification of the TME [50, 86, 87]. Additionally, type 2 pericytes have also been associated with tumorigenesis and vascular formation for tumors [88]. Pericytes recruited for vascular formation have been associated with ERα expression and E2-dependent signaling during function [89, 90].

2.3.3 Cancer-Associated Fibroblasts

CAFs are one of the most integral stromal cell types in the TME for tumor survival and metastasis via paracrine-induced signaling pathways via chemokines and soluble growth factors [91, 92]. ERα expression in breast CAFs have been observed in vivo through nuclear receptor arrays comparing gene expression between CAFs and normal human breast adipose fibroblasts [53]. Interestingly, similar levels of ERα expression are seen in both malignant and physiologic fibroblasts, but with downstream upregulation of the direct transcriptional activator liver receptor homolog-1 (*LRH-1*) in the former [53]. The regulator serves to increase expression of the aromatase-encoding gene *CYP19A1* [93–95]. Co-expression of aromatase and LRH-1 in the breast TME suggests CAF-induced paracrine formation of E2 and subsequent ER-mediated oncogenesis [96]. Coculturing of endometrial CAFs with endometrial neoplastic cells have been seen to contribute to tumor progression, possibly attributed to CAFs' expression of ERα and ERβ [56]. This tumor progression mechanism is supported through in vitro upregulation of phosphatidylinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling networks, which are both well-known ER-mediated pathways in breast and lung cancer [56, 97–99].

Contrastingly, ER expression in prostate CAFs has contradicting evidence, with reports of ERα/ERβ expression portending advanced disease [54] and others suggesting ERα expression is a protective factor again neoplastic invasion macrophage infiltration [100, 101]. These latter in vitro studies conveyed that CAF ERα expression reduced murine and human prostate cancer cell invasion, as well as lymph node metastasis of orthotopically implanted human prostate cancer cells in mice [101]. These ERα-positive CAFs appeared to halt invasion and metastasis of human prostate cancer cells through downstream downregulation of the C-C motif chemokine ligand 5 (CCL5) and IL-6 chemokines, whose roles are involved in growth factor signaling, inflammation, and tumor recruitment [102, 103].

2.3.4 Tumor-Associated Macrophages

In a physiologic setting, macrophages regulate tissue-specific innate immune responses to fight foreign invaders through polarization by varied cytokines. However, TAMs have been complicit in tumor proliferation and migration, as well as inflammation in the TME [104, 105]. Physiologically, polarized M1 macrophages secrete the proinflammatory cytokines IFNγ, interleukin 12 (IL-12), and tumor necrosis factor (TNF)-α for tumor rejection and antigen presentation [106]. Alternatively, M2 macrophages produce interleukins 4, 5, 6, and 10 [106], which are known promoters of tumor cell growth and immune evasion [107]. TAMs within the TME

are often M2, with denser concentration demonstrating worse OS, thus offering a therapeutic opportunity for a variety of malignancies [108].

TAMs are an independent poor prognostic predictor for ovarian adenocarcinoma [109]. Relatedly, co-localized expression of both ERα/ERβ is reported in human high-grade serous ovarian cystadenocarcinoma (HGSOC) TAMs. Interestingly, HGSOC in premenopausal women demonstrates elevated TAM infiltration relative to that of postmenopausal women. The highest concentration of TAMs in this TME can be found in ERα-positive tumors [58]. The mechanism of this was elucidated by an IHC analysis revealing aromatase expression in the TME of breast TAMs, which was observed to increase E2 production and breast cell proliferation [59]. TAM proliferation, however, is relatively more prevalent in ER-negative breast malignancies [110, 111]. It is important to note, however, that quantification of TAM polarization was not analyzed in these studies. Interestingly, aromatase and ERβ expression in NSCLC TAMs have also been observed, specifically in infiltrating macrophages of preneoplastic, tobacco carcinogen-induced murine lung lesions [17, 60].

Although direct observation of ER expression in TAMs has been limited, E2 induction of M2 polarization and subsequent tumor spread has been studied. A polyomavirus middle T (PyMT), ER-positive breast cancer murine model demonstrated direct E2 stimulation of tumoral M2 TAM infiltration and vascular endothelial growth factor (VEGF) [112, 113]. Alternatively, untreated controls exhibited M1 TAM infiltration instead [112]. In a HGSOC, ovariectomized murine model, E2 induced growth of both ER-negative xenografts and M2 TAM infiltration [58]. In tobacco carcinogen-exposed mice, administration of E2 increased pulmonary TAM infiltration while mice receiving the aromatase inhibitor anastrozole had a significant reduction in pulmonary TAMs [114]. Further, E2-induced VEGF expression was also observed in this model [114]. Of note, E2-mediated TAM infiltration has been observed in vitro to be fed forward via M2 TAM-induced epigenetic ERα upregulation via interleukin 17A (IL-17A) in endometrial malignancy [115]. This positive feedback mechanism between E2 and M2 TAMs provides a potential therapeutic target, a concept recently addressed via effects of the phytoestrogen SERM resveratrol in a lung cancer xenograft model [116]. Resveratrol treatment appeared to suppress tumor proliferation through decreased signal transducer and activator of transcription 3 (STAT3) signaling and M2 polarization [116].

2.3.5 Myeloid-Derived Suppressor Cells

MDSCs are another myeloid cell present in the TME known to interfere immune surveillance and facilitate tumor growth [117]. ERα expression in human ovarian adenocarcinoma MDSCs has been identified by IHC and confirmed through PCR and immunoblotting [57]. In an E2-insensitive syngeneic ovarian cancer model, ovariectomized mice exhibited improved survival compared to non-ovariectomized mice following tumor challenge. Contrastingly, E2 supplementation in these mice accelerated tumor progression and reversed the protective effect found in estrogen-depleted mice [57]. Of note, this study found that T-cell-deficient mice lost survival benefit of estrogen depletion, suggesting adaptive immunocompetence to be mechanistically integral [57]. Estrogen's effect on the two legs of immunity was also observed in E2-treated mice, which were found to have notably decreased concentrations of helper and cytotoxic T cells, and significantly increased concentrations of granulocytic MDSCs in spleen and tumor beds [57]. ER-dependence of MDSC expansion was further studied with in vitro administration of the ERα antagonist methylpiperidino pyrazole (MPP) to inhibit MDSC proliferation [57]. Ovarian tumor-bearing mice treated with E2 had measurable JAK2 and SRC upregulation with downstream STAT3 signaling, a regulator of myeloid differentiation and development [118]. In syngeneic lung and breast cancer murine models, E2-stimulated tumor growth was mitigated by MDSC depletion after treatment with anti-Gr1 antibodies [57, 119, 120]. Patients with cervical cancer that were pregnant with high E2 had increased expansion

of MDSCs and shorter PFS. These findings were further evaluated in mouse models [120].

2.3.6 Inflammatory Cytokines and Eicosanoids

Chronic inflammation has been accepted as a common factor in tumorigenesis and spread. TME facilitates neoplastic progression primarily through cytokine-induced oncogenic pathway activation, leading to cell proliferation, immune evasion, and infiltration [121]. IL-6 from TAFs has been observed to assist ERα-positive breast cancer proliferation and immune evasion [122] via STAT3 activation in vitro and in vivo [123]. TNFα in ERα-positive breast cancer cells has been observed to regulate gene expression for metastasis [124]. This cytokine has also been shown to upregulate aromatase expression in cultured human adipose stromal cells [125]. Neoplastic implication of these inflammatory markers is evidenced by data showing TNFα and IL-6 correlate closely with aromatase expression in human breast cancer tissue and not in adjacent noncancerous tissue [126]. Aromatase has similar transcriptional correlation with cyclooxygenase-2 (COX-2) [126]. COX-2 mediates the inflammatory response by producing eicosanoids such as prostaglandin E2 (PGE2) [127], which upregulates aromatase expression through cyclic adenosine monophosphate (cAMP) in breast malignancy [128]. Despite conflicting reports, a case–control study demonstrated regular administration of the nonsteroidal anti-inflammatory drug (NSAID) aspirin reduced the risk of developing ERα-positive breast cancers (hazard ratio (HR) = 0.74; 95% CI, 0.60–0.93), but not ERα-negative cancers (HR = 0.97; 95% CI, 0.67–1.40) [129].

ERα, TNFα, and NF-κB protein expression correlate closely in breast cancer tissues [130]. NF-κB signaling, a proinflammatory cytokine associated with IL-6 and TNFα, is often constitutively activated in many tumor types [131]. High levels of the cytokine are also implicated in SERM resistance in ERα-expressing human breast cancer cells [132, 133]. E2 also enhanced pulmonary inflammation through increased NF-κB, VEGF, and IL-17A in a murine model evaluating tobacco carcinogen-induced lung cancer [114]. E2 inhibition with combined AI/NSAID treatment served to noticeably decrease pulmonary malignancy in these mice. Notable pathways affected included IL-17A expression, IL-6 concentration, as well as STAT3 and MAPK [114]. Cumulatively, there appears to be a potential target for the E2 pathway as it interacts with tumorigenesis via inflammation.

2.3.7 The Impact of Supraphysiologic Estrogen

Esterified estrogen, specifically estrone, is significantly increased in the setting of obesity. Aromatase in adipocytes serves to increase estrone secretion in the setting of hypertrophy. The effect this supraphysiologic estrogen has on tumorigenesis has been controversial [134, 135]. Recent findings suggest that while immune dysfunction and tumor progression are associated with obesity, improved response to immunotherapy may also be associated with obesity, supporting the immune-mediated link between obesity and cancer [136]. Chronic inflammation from obesity is integral to carcinogenesis and tumor evolution, as observed in postmenopausal, ER- and progesterone receptor (PR)-expressing breast malignancy [137]. It is important to note that studies suggesting protumor effects of estrogen in estrogen-depleted mammals have been performed primarily in the setting of hormone replacement therapy (HRT).

Tumorigenesis, progression, and infiltration in the setting of HRT in estrogen-depleted mammals remain controversial. There is a paucity of studies demonstrating proinflammatory changes with hormone replacement therapy in murine models. In contrast, there are many studies conveying a protective effect of exogenous estrogen. Specifically, ERβ-expression has been observed to prevent progression of human colorectal carcinoma (CRC) [134, 138]. Mechanistic protection against carcinogenesis with exogenous estrogen in postmenopausal patients appears to primarily

be through a decrease in the natural postmenopausal increase in Th1/Th2 ratio [139, 140]. Specifically, Th2 cytokines are quantifiably stable until late postmenopausal stage, while production of Th1 cytokines is progressively increased in women after menopause. HRT prevents this increased Th1/Th2 ratio, thereby improving the aberration of Th1/Th2 balance that is implicated in an inadequate immune response and neoplastic conditions [140]. Substantiation of this antitumoral concept was provided through an in vivo, placebo-controlled study regarding postmenopausal human breast cancer cell demonstrating estrogen's notable decrease in IL-6 production [141].

2.4 Clinical Implications of Targeting the Estrogen Pathway in the Tumor Microenvironment

Immunotherapy is a developing and effective treatment avenue in the world of cancer; yet the TME and its immunosuppressive mechanisms is a deterrent for large-scale success. As it stands, the immune checkpoint modulators of cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and PD-1/PD-L1 are the most studied immunotherapies [142]. These revolutionary options have had dramatic impacts on OS relative to standard-of-care chemotherapies [143–146]. Even so, response rates are limited to 20–35% of cases, closely dependent on tumor type, stage, and PD-L1 expression [147]. Moreover, 25–33% of melanoma patients often demonstrate delayed relapse during treatments attributed to tumor cell adaptation [5, 6].

There appears to be a balance of tumoral mutations and immunoediting that facilitate immune evasion, and subsequently, failure of checkpoint therapy. On the one hand, damaged DNA repair mechanisms, increased non-synonymous somatic mutational load, and neoantigen presentation cripple immune evasion and improve OS [2, 3, 148]. On the other hand, damage to antigen-presenting mechanisms, as well as recurrence of nonantigenic mutations, appears to facilitate immune evasion [149, 150]. Studies identifying these mechanisms provide insight into measurable biomarkers to assess tumor responsiveness to current and, inevitably necessary, novel immunotherapies. A potential investigative therapy is endocrinological agents that modulate E2 and its protumoral pathway to abrogate tumor immune evasion. Specifically, anti-estrogen therapy may reduce TME immunosuppression while increasing E2-sensitive tumor responsiveness.

Recently, a high-throughput screening assay in human lung cancer cells demonstrated fulvestrant, an anti-estrogen agent, as the most efficacious compound in increasing tumor sensitivity to immune-mediated lysis [151]. Fulvestrant additionally has few interactions and overlapping toxicities with anti-PD-1/PD-L1 agents. Thus, anti-E2 therapies to target the immunosuppressive TME could increase efficacy and duration of response of current immune checkpoint inhibitors (ICI) [119, 152] (Fig. 2.1).

Based on the well-established evidence of sex-driven dimorphism in immune function and response, patient sex has been postulated to have an influence on the efficacy of ICIs [9]. This sexual dimorphism plays an important role in the disparity of cancer immunoediting in females and males and could not only explain differences in progression and mortality observed between male and female cancer patients but also sex differences in response rates, toxicity patterns, and outcomes to treatment with ICIs. In support of this concept, the PD-1/PD-L1 pathway is modulated by multiple X-linked microRNAs (miRNAs), which crosstalk with the estrogen-ERα axis, suggesting an important role of the estrogen pathway and response to ICIs [153, 154]. Further since estrogen modulation of the PD-1/PD-L1 pathway has been demonstrated in animal models [82, 155], it is reasonable to expect that immunotherapy efficacy may vary according to patient sex.

In an effort to identify patient characteristics linked to ICI effectiveness, several meta-analyses have been conducted to evaluate sex-differential effects in efficacy of ICIs. Conforti et al. evaluated the effect of patient's sex on the efficacy of

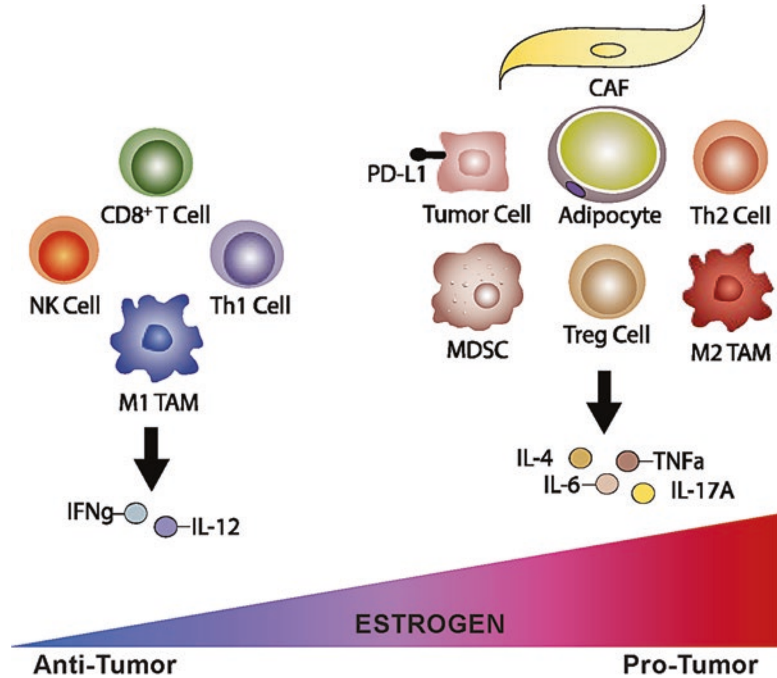

Fig. 2.1 Increasing estrogen promotes a pro-tumor TME via increased Th2 responses, increased production of tumor-promoting cytokines (IL-4, IL-6, TNFα, and IL-17A), M2 TAM infiltration, decreased Th1 cytokines (IL-12 and IFNγ), and M1 TAM infiltration. E2 has also been associated with increased Treg and MDSC proliferation, increased PD-L1 expression on tumor cells, and decreased CD8+ T cell and NK cell proliferation. CAFs and adipocytes may also serve as pro-tumor as they can supply E2 and IL-6. (Adapted from [64])

ICIs measured in terms of OS on different tumor types [156]. This study included 11,351 patients (67% men and 33% women) enrolled in 20 Phase II and III randomized controlled trials that evaluated CTLA4 inhibitors, as well as PD-1/PD-L1 inhibitors in patients with different tumor types, mostly melanoma and NSCLC. Results showed that male patients who received ICIs alone had a reduced risk of death compared to men in the control arms (HR = 0.72, 95% CI 0.65–0.79). Similar findings were observed in female patients, but the difference in risk reduction was smaller between the treatment and the control arm (HR = 0.86, 95% CI 0.79–0.93). Although there was a significant difference in the efficacy of ICIs between male and female patients, the heterogeneity test for this sex-related interaction was not quite significant.

A subsequent meta-analysis evaluated the differences in outcomes based on sex in lung cancer patients who received targeted therapy or immunotherapy [10]. This study included a total of 12 Phase III clinical trials evaluating EGFR, ALK, and PD-1 inhibitors versus chemotherapy. Of the 12 trials included in this meta-analysis, five compared PD-1 inhibitors versus chemotherapy, two of which compared pembrolizumab versus chemotherapy (KEYNOTE 010 and KEYNOTE 024), and three compared nivolumab versus chemotherapy (CheckMate 017, CheckMate 026, CheckMate 057) [144, 157–159]. The studies that compared ICIs versus chemotherapy included 1028 female and 1435 male lung cancer patients. While there was significant heterogeneity between STUDIES, OS was favorable in male patients treated with ICIs compared to chemotherapy (HR = 0.76; 95% CI 0.068–0.86; $p < 0.00001$). There was no significant difference in survival in female lung cancer patients receiving chemotherapy compared to ICIs (HR = 1.03; 95%CI 0.89 to 1.03; $p = 0.69$). In a separate study focused on metastatic NSCLC, El-Ostra et al. evaluated results from eight randomized clinical trials for predictors of benefit to single agent ICIs over chemotherapy [8]. NSCLC patients treated with ICIs had significant progression-free survival (PFS) superiority in ever-smokers, male patients, and patients with PD-L1-positive tumors. In contrast, female NSCLC patients had comparable PFS between ICIs and chemotherapy.

Wallis et al. also conducted a meta-analysis that included 23 randomized clinical trials (67.9% men and 32.1% women) that compared ICIs (both ICI alone and ICI plus chemotherapy trials) to standard-of-care treatment in advanced solid tumors (including NSCLC, SCLC, urothelial carcinoma, head and neck squamous carcinoma, melanoma, mesothelioma, clear cell renal carcinoma, and gastric or gastroesophageal carcinoma). In this study, no difference in OS between men and women who received immunotherapy was observed ($I^2 = 38\%$; $p = 0.6$) [160]. The conflicting results and limitations in these meta-analyses suggest that further investigation of the efficacy of ICIs and patients' sex is warranted in future studies. While the majority of the trials included in these studies were underpowered to detect clinically relevant sex differences in outcome, these results indicate that the hormonal milieu may have some effect on treatment response (Table 2.2).

The current best predictive markers of therapeutic response to ICIs are high PD-L1 expression and high tumor mutational burden (TMB). The difference between PD-L1 expression between men and women has been evaluated in some cancer patient cohort with a reported increased PD-L1 expression in male patients [161–163]. TMB has also been shown to be lower in women compared to men ($p = 0.0349$), across multiple studies [164, 165]. TMB is predictive of response to ICI in lung cancer and is lower in female lung cancer patients compared to male lung cancer patients [165]. Similarly, sex differences in immune-related adverse events (irAEs) have also been noted in ICI trials [166, 167]. The gut microbiome and obesity are emerging areas of interest that may predict response to ICIs [168]. Whether or not these factors interact with sex hormones in the context of anti-cancer immunity is yet to be determined.

2.5 Conclusions and Perspective

The E2 pathway is an identified promoter of tumorigenesis in several cancers, largely for its genomic, epigenomic, and transcriptional effects on tumor cells and the TME. The reciprocal interactions of the peritumoral and tumoral environment are becoming more evident, with E2 playing a major role in modulation of primarily protumoral pathways. With immunoediting being a culprit in E2-mediated protumoral activity, it appears to be an important deterrent for checkpoint blockade immunotherapy success. Thus, inhibition of the E2 pathway may augment current immunotherapy response rates.

Carcinogenesis from obesity and its related illnesses are thought to be primarily driven through proinflammatory cytokine secretion. Supraphysiologic estrogen from adipocyte aromatase expression may also play a role, but as of now, it is difficult to distinguish. However, estrogen replacement therapy in postmenopausal women appears to have a relatively protective effect via immune modulation. Stabilization of immunologic aberrancies, notably in the adaptive immune system, is protective against age-related malignancies such as colorectal carcinoma and breast cancers. Based on the above discussion, future studies are war-

Table 2.2 Selected trials evaluating the combination of Estrogen pathway targeting agents with ICIs

Malignancy	Selected study drugs	*n* =	Clinical trial number
ER+/Her2- Breast cancer	Exemestane and durvalumab/tremelimumab	240	NCT02997995
ER+/Her2- Breast cancer	Pembrolizumab, letrozole, and palbociclib	22	NCT02778685
ER+/Her2- Breast cancer	Atezolizumab and fulvestrant	126	NCT03280563
ER+/Her2- Breast cancer	Pembrolizumab and exemestane	25	NCT02990845
ER+/Her2- Breast cancer	Pembrolizumab and AI	37	NCT02971748
ER+/Her2- Breast cancer	Pembrolizumab and letrozole, exemestane anastrozole	56	NCT02648477
AR+/ER- Breast cancer	Pembrolizumab and enobosarm	29	NCT02971761

Selected ongoing trials evaluating ICI in combination with therapeutic agents targeting the E2 pathway. Disease type, selected study agents, predicted accrual size, and clinical trial number are provided

ranted to assess responsiveness to current ICIs across sex, menopausal status, and BMI in order to isolate E2 pathway contribution to immune evasion.

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3 The Non-Bone-Related Role of RANK/RANKL Signaling in Cancer

Peter A. van Dam, Yannick Verhoeven, and Xuan B. Trinh

Abstract

RANK ligand (RANKL) is a member of the tumor necrosis factor alpha superfamily of cytokines. It is the only known ligand binding to a membrane receptor named receptor activator of nuclear factor-kappa B (RANK), thereby triggering recruitment of TNF receptor-associated factor (TRAF) adaptor proteins and activation of downstream pathways. RANK/RANKL signaling is controlled by a decoy receptor, osteoprotegerin (OPG), but also has additional more complex levels of regulation. It is crucial for the differentiation of bone-resorbing osteoclasts and is deregulated in disease processes such as osteoporosis and cancer bone metastasis. Cells expressing RANK and RANKL are commonly found in the tumor environment. In many tumor types, the RANK/RANKL pathway is overexpressed, and this is in most cases correlated with poor prognosis. RANK signaling plays an important role in the innate and adaptive immune response, generates regulatory T (Treg) cells, and increases the production of cytokines. It is also involved in chemo resistance in vitro. Recent evidence suggests that RANKL blockade improves the efficacy of anti-CTLA-4 antibodies against solid tumors and experimental metastasis. Therefore, there is increasing interest to use RANKL inhibition as an immunomodulatory strategy in an attempt to make immune-resistant tumor responsive to immune therapy.

Keywords

RANK · RANKL · Osteoprotegerin · Microenvironment · Cancer · Bone health · Immunomodulation · PFD-L1 · CTLA-4 · Immune response · Inflammation · Immune tolerance · Angiogenesis

3.1 Background

In most cancer types, only a minority of patients have an improved survival after immune therapy. Mutational burden, neoantigen load, quality and clonality of neoantigens, expression of antigen presenting molecules and immune checkpoints, interferon gamma responsiveness, and composition of the microenvironment (hot versus cold tumors), all influence the beneficial effects of

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immune therapies [1]. Although combinations of immune therapy (e.g., CTLA-4 and PD-L1 blocking) can be synergistic, they do not resolve their diminutive effectiveness in cancer treatment and often induce significant additional toxicity [2–5]. Hence, there is an increasing interest in combining immune therapy with less toxic immune modulating drugs to sensitize immune unresponsive tumors to immune therapies [6]. Recent data suggest that RANK/RANKL inhibition may be an attractive approach to increase the effectiveness of immunotherapy. Signaling between the receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL) is essential for the differentiation of bone-resorbing osteoclasts and is deregulated in pathological processes such as postmenopausal osteoporosis or cancer-induced bone destruction [2]. However, cells expressing RANK and RANKL are also commonly found in the tumor microenvironment. RANK signaling plays an important role in the innate and adaptive immune response as it generates regulatory T (Treg) cells and increases production of cytokines [7, 8]. In this chapter, the effects of RANK/RANKL signaling inhibition on the microenvironment of malignant tumors are reviewed. It is hypothesized that this approach may be used to improve the response to immunotherapy (Fig. 3.1).

3.2 The RANK/RANKL Signaling Pathway

The receptor activator of nuclear factor-kappa B ligand (RANKL) was originally identified in T cells and dendritic cells (DC) [2]. It is a type II homotrimeric transmembrane protein that has three known isoforms. RANKL1 and RANKL2 are expressed as membrane bound proteins. RANKL3 is a soluble secreted protein that is formed by cleavage of the membranous counterparts or by alternative splicing [9]. The RANKL has a large cytoplasmic domain containing four cystein-rich repeat motifs and two N-glycosylation sites. The full length RANKL is called RANKL1, in RANKL2 a part of the intracellular domain is deleted, while in RANKL3 the N-terminal part misses [7, 10]. The RANKL is encoded by the TNFS11 gene in humans and is also named osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPGL), or TNF-related activation induced cytokine (TRANCE) [9, 11]. It is the only known ligand binding to a membrane receptor named receptor activator of nuclear factor-kappa B (RANK), which is a type I transmembrane protein belonging to the TNF receptor superfamily (TNFRSF11A) [11, 12]. Binding between RANKL and RANK induces

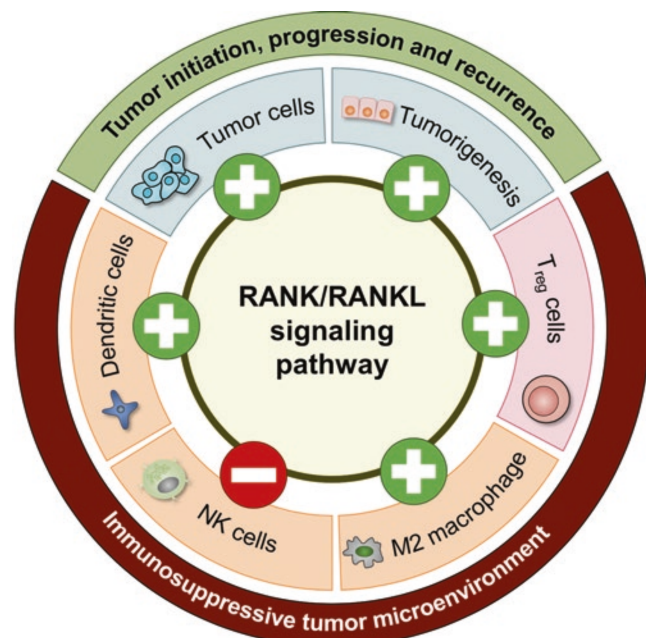

Fig. 3.1 Main effects of RANK/RANKL signaling pathway on tumor growth, immune cells, and microenvironment

trimerization of the receptor. This triggers recruitment of TNF receptor-associated factors (TRAF), adaptor proteins, and activation of downstream signaling pathways (such as NF-kB, AKT/PKB, JNK, and the MAP kinase cascade) [7, 13, 14]. A regulatory system is built into the RANK/RANKL signaling pathway by means of a decoy receptor called osteoprotegerin (OPG, TBFRSF11B) interacting with RANKL [2]. OPG is a soluble glycoprotein that can exist either as a 60-kDa monomer or as a 120-kD dimer but lacks transmembrane or cytoplasmatic domains. The dimerization of OPG increases the affinity of OPG to RANKL dramatically and is essential for RANK/RANKL signal inhibition [15]. Several factors can upregulate OPG expression such as estrogen (which is important for bone metabolism), TRAIL, Wnt, and TNFalpha [7]. On the other hand, it can be downregulated by PTH and TGF-beta [9]. The overall inhibitory effect of OPG on RANKL depends on the balance of its binding to these various ligands [11, 12]. The RANK/RANKL signaling network is further complexed by a second, more recently discovered, decoy receptor for RANKL, LGR4 [14]. LGR4 suppresses canonical RANK signaling by competing with RANK to bind RANKL. The binding of RANKL to LGR4 activates the Gαq and GSK3-β signaling pathway. This suppresses the expression and activity of nuclear factor of activated T cells and calcineurin-dependent 1 (NFATC1) during osteoclastogenesis. Furthermore, functional RANK splicing variants have also been identified, implicating several sophisticated levels of the pathway [16].

3.3 The Functional Role of RANK/RANKL Signaling in Humans

RANK and RANKL can be detected in many different tissues, such as the bone, prostate, thymus, mammary glands, and liver, implicating a functional role in these organs [17]. Studies in mice indicate that RANK/RANKL signaling is required for mammary gland development and lymph node formation [7, 17, 18, 19]. The signaling pathway's crucial role in healthy bone remodeling and bone homeostasis is, however, much better documented [8]. The RANK/RANKL pathway regulates the formation of multinucleated osteoclasts from their monocyte-macrophage precursor cells and subsequently also their activation and survival [8]. By binding RANKL, OPG prevents it to connect to and activate RANK, thereby protecting the skeleton from excessive bone resorption [19, 20]. When deregulated, this pathway may lead to pathological processes such as cancer-induced bone destruction and osteoporosis but also chronic inflammatory processes such as inflammatory bowel diseases and arthritis [14, 20, 21].

Another well-known functional role for RANK/RANKL signaling is that of modulating the immune response. RANK/RANKL and OPG knockout mice showed a disrupted immune phenotype (e.g., impaired T or B cell development) [7, 22]. RANKL can be found in tumor-infiltrating lymphocytes (TILs), immature dendritic cells, B cells, macrophages, and monocytes [2]. RANK activation induces lymphocyte differentiation, T-cell activation, and dendritic cell (DC) survival, triggering intracellular signaling pathways (e.g., MAPK, NFkB, p38, and c-JNK) and even extracellular kinases (ERK) [17, 23, 24, 25]. RANKL can induce the expression of multiple activating cytokines by DCs, including IL-1, IL-6, IL-12, and IL-15, and can enhance DC survival via the induction of the antiapoptotic protein Bcl-xL (B-cell lymphoma-extra large) [2]. Dendritic cells prime and activate T cells during the immune response by processing and presenting antigens to them. The RANKL signal can alter the function of dendritic cells, which may lead to an increase of Foxp3-positive Tregs [7]. Recent evidence suggests that in response to injury, pericytes are also able to modulate local tissue immune responses via several independent pathways including RANKL signaling. In this area, the OPG/RANK/RANKL axis in association with the functions of pericytes may be involved in vasculogenesis, the process of atherosclerosis by altering lipid metabolism, vascular signaling, and angiogenesis [26, 27].

3.4 RANK/RANKL Signaling in Cancer

Several studies documented RANK signaling to be important in a variety of cancers [23–43]. This was recently nicely reviewed by Renema et al. and de Groot et al. [17]. Tregs are a CD4+ helper T-cell subset that can suppress autoimmune responses in the body and are critical to create an immune suppressive environment in cancers [2]. Together with other partners, such as TAMs, they can create a status of local immunosuppression surrounding the tumor [7]. TAMs express immune checkpoint modulators (such as PD-L1) that directly inhibit activated T cells and produce various chemokines that attract other immunosuppressive cells, such as Tregs and myeloid-derived suppressor cells (MDSCs) [11]. In many situations, the RANK network is an important driver to create an immunosuppressive microenvironment, thereby promoting tumor progression. The central role of RANK/RANKL signaling in bone metastasis has been well studied [9]. The RANK signal network has been shown to drive epithelial to mesenchymal transition (EMT), induce stem cell-like phenotypes, promote osteomimicry, and give cancer cells the ability to home to bone [11, 39]. In a large population of breast cancer patients, strikingly high levels of RANK expression in the primary tumor were predictive for the frequency of the later occurrence of bone metastasis [37]. Recently, it seems that RANK signaling is important in the biology of many tumor types beyond bone metastasis [2, 7, 8, 11, 17, 23–43]. RANK and RANKL-expressing cells are commonly found in the tumor microenvironment [2]. The RANKL/RANK pathway is often overexpressed in cancers of the prostate, endometrium, stomach, breast, cervix, stomach, bladder, oesophagus, and thyroid, which is correlated with poor prognosis [23–43]. RANKL has been detected in endothelial cells and implicated in angiogenesis [7].

There is some circumstantial evidence suggesting that paracrine signaling through RANK/RANKL is responsible for the expansion of mammary stem cells observed during pregnancy and luteal cycles [13, 38]. MMTV-RANK transgenic mice are prone to develop mammary tumors, which may be related to activated RANK signaling [35]. Pharmacologic inhibition of RANKL or genetic ablation of RANK reduces (particularly estrogen and progesterone receptor negative) mammary tumor and metastasis development in animal models [32]. Breast cancer cells are able to produce RANKL and stimulate osteoclast differentiation [16, 38, 39]. In humans, high RANK expression is associated with altered mammary differentiation, which suggests that increased RANK signaling may contribute to breast carcinogenesis [13, 40]. High RANK expression was particularly detected in human primary breast adenocarcinomas that lack expression of the hormone receptors, in tumors with high pathologic grade and proliferation index. It is associated with the presence of metastases and poor prognosis [37]. It has been shown in vitro that HIF-1 alpha-induced expression of RANKL initiates increased migration of breast cancer cells via PI3K/AKT signaling, illustrating that the RANK/RANKL pathway also plays an important role in breast cancer progression [41, 42].

Mouse models and randomized studies in humans have shown that combination of antibodies blocking OPG or RANK with chemotherapy, hormone therapy, or targeted drugs resulted in stronger decrease of tumor burden in the bone [8, 17]. However, inhibition of RANK signaling also has a direct effect on tumor cells at other locations [28]. The RANK/RANKL pathway was variably expressed in tumors of the thyroid, and increased serum OPG was also correlated with poor prognosis in gastric, cervical, esophageal, and bladder carcinoma [23–27, 29–32]. Song et al. found that RANK expression was significantly higher in hepatocellular carcinoma (HCC) than in peritumoral hepatic tissue [33]. HCC cell lines express RANK constitutively, and activation of the RANK-RANKL axis significantly promoted migration and invasion ability of HCC cells in vitro. Recently, it has been demonstrated that RANK/RANKL expression is also significantly elevated in endometrial and prostate cancer tissue, particularly in tumors of higher stage [20, 34, 35, 44]. Therefore, there may be a role for RANKL inhibitors as a therapeutic strategy.

3.5 Effects of the RANK/RANKL Signaling Pathway on the Tumor Microenvironment

RANK and RANKL expressing cells are commonly found in the tumor microenvironment [20]. RANKL modulates the immune response by inducing T-cell proliferation and dendritic cell survival [45]. In human breast carcinomas, RANKL is found in tumor-infiltrating lymphocytes (TILs), and RANK is strongly expressed in tumor-associated macrophages (TAMs) [18]. TAMs accumulate in the microenvironment and, depending on their M2 or M1 phenotype, are involved in tumor growth, angiogenesis, and metastasis. RANKL acts as a chemoattractant for these cells [2]. RANK/RANKL signaling in M2 macrophages modulates production of chemokines, promoting the proliferation of Tregs and thereby creating an immunosuppressive environment. As RANKL is mainly produced by Tregs, a vicious circle is established in conjunction with the TAMs mainly expressing RANK [7, 46]. Tumor-infiltrating Tregs have been shown to stimulate mammary cancer metastasis through RANKL-RANK signaling [47]. RANKL treatment enhances survival of mature dendritic cells (DCs) and triggers generation of proinflammatory cytokines (IL-1, IL-6, and IL-12) that can promote differentiation of CD4+ T cells into Th1 cells, providing a major costimulatory factor for CD4+ T-cell responses [47]. RANK is also expressed on NK cells, playing an important role in immunosurveillance. RANKL/RANK is involved in crosstalk between the bone and the immune system. It stimulates osteoclasts to function as antigen-presenting cells, thereby activating CD4+ and CD8+ T cells. A similar phenomenon might also be present in the microenvironment of solid tumors [7]. The crosstalk of tumor cells with the immune system is not completely understood, but the impact of RANK-RANKL signaling on the tumor immune response is likely to be context specific [4]. Due to sequestering OPG by tumor cells or entrapment of OPG by the proteoglycans and glycosaminoglycans of the extracellular matrix, a microenvironment is created that facilitates the expansion of the tumor cells [48]. In addition, OPG can block TRAIL activity, thereby acting as an antiapoptotic and pro-proliferative stimulus for cancer cells [11, 21]. It has been shown that RANK/RANKL signaling can promote the initial stages of cancer development by inducing stemness and epithelial-mesenchymal transition [19]. RANKL (e.g., produced by osteoblasts or bone marrow stromal cells) attracts RANK-expressing cells and induces their migration by activation of specific signaling pathways, such as the MAP kinase pathway [44]. RANKL was also detected in endothelial cells and has been implicated in angiogenesis through Src and phospholipase C-dependent mechanisms [2, 49].

3.6 RANKL Signaling Inhibition

The only commercially available inhibitor of RANKL is denosumab. This drug is a fully human monoclonal antibody that binds RANKL, thereby blocking its interaction with RANK [2, 8]. Denosumab is approved by the Food and Drug Administration for the treatment of osteoporosis and giant cell tumor of the bone and for the prevention and treatment of skeletal complications caused by bone metastases and lytic bone lesions in multiple myeloma [8]. The drug has a well-known and acceptable toxicity profile [8]. It remains unclear whether RANK/RANKL inhibition with denosumab in patients with cancer has any effect beyond the bone. In a post hoc analysis of patients with non-small-cell carcinoma of the lung (NSCLC) that were included in a phase III randomized trial comparing zoledronic acid versus denosumab, a survival benefit was observed (HR 0.80; 95% CI 0.67–0.95, $p = 0.01$) for the patients treated in the denosumab arm [50]. There was no difference in the delay of bone events in both groups, and the beneficial effect of denosumab could be observed in patients with visceral metastasis, as well as in patients with bone metastasis only. However, the recent prospective SPLENDOUR trial could not show any improvement in OS or PFS by adding denosumab to standard first-line therapy in patients with metastatic NSCLC [51].

The effect of adjuvant denosumab in women with early breast cancer was recently studied in two large, multicenter, prospective, randomized trials [52, 53]. In the ABCSG-18 study, it was shown that disease-free survival was significantly better in the denosumab group [52]. This study compared placebo or denosumab 60 mg subcutaneously every 6 months for 5 years in 3425 postmenopausal patients with hormone-sensitive early breast cancer treated with an aromatase inhibitor. In the DCARE study, which assessed 4509 high-risk early breast cancer patients treated with standard therapy either with or without denosumab 120 mg SC every month (for 6 months, then 3 monthly up to 5 years), no improvement in bone metastasis-free, disease-free, or overall survival was reported, even though there was an improvement in time to bone metastasis at site of first recurrence in the denosumab group [53]. It is important to mention that most (95.9%) of these patients had received taxane or anthracycline-based chemotherapy. This raises the hypothesis that chemotherapy may reduce some of the tumor suppressive effects of RANK/RANKL inhibition in the cancer microenvironment. Other explanations may be the differences in molecular characteristics of the tumors of these patient populations, or effects of the menopause and endocrine treatment on the tumor behavior. It is clear that more research is necessary to unravel the effect of denosumab on tumor behavior. In the D-BEYOND trial, the biological effects of two neoadjuvant injections of 120 mg denosumab (1 week apart) in 27 patients with premenopausal primary breast cancer were evaluated [54]. The authors concluded that 2 weeks of RANKL inhibition did not have an effect on the tumor proliferation rate, but significantly increased the number of TILS in the tumor environment, making them theoretically more susceptible for immune therapy. Recently, some additional evidence emerged that RANK/RANKL inhibition may have a role as immune modulator. In preclinical studies, RANKL blockade improves the efficacy of anti-CTLA-4-targeted antibodies in solid tumor models of metastasis [53]. Bakhru et al. showed that antibodies blocking RANKL and CTLA-4 cooperate to increase the frequency of tumor-infiltrating CD4+ T cells expressing cytolytic markers, thereby improving antimelanoma immunity [55]. Addition of RANKL blockade to anti-PD-1 and anti CTLA-4 resulted in superior tumor responses and was most effective if RANKL inhibition was given concurrent or following checkpoint blockade [54]. This triple combination therapy improved T-cell effector function in tumor bearing mice by increasing the proportion of tumor-infiltrating CD4+ and CD8+ T cells that can produce both interferon gamma and TNF. In 2014, Smyth et al. described a case of a rapidly advancing metastatic melanoma with aggressive and symptomatic bone metastases requiring treatment with the anti-RANKL antibody denosumab for palliation in a patient who was concomitantly treated with ipilimumab (an anti-CTLA-4 antibody) [56]. She had a spectacular partial response and was alive at 62 weeks. In a melanoma preclinical model, these authors could demonstrate that monoclonal antibodies (mAbs) directed to CTLA-4 or RANKL have modest antimetastatic activities in monotherapy, but when these drugs were combined at the time of intravenous melanoma inoculation, the development of metastases was significantly reduced. Mechanistically, the combined effect of anti-CTLA-4 and anti-RANKL depends on lymphocytes or natural killer cells. In a retrospective study, Afzal and Shirai evaluated the synergistic effect of immune checkpoint inhibitors and denosumab in metastatic melanoma patients [57]. Eleven (29.72%) out of 37 patients were treated with immune checkpoint inhibitors and denosumab, and the others only immune checkpoint inhibitors. The median progression-free and overall survival in the cohort having the combination treatment, respectively, was 11.6 and 57 months compared with 4.15 and 22.8 months in the control group. Although there are potential confounders, this suggests that adding denosumab to immune checkpoint inhibitors may have a beneficial effect on outcome. In a subsequent study, Ahern et al. assessed the efficacy of a combination of RANKL and CTLA-4 blockade by analysis of tumor-infiltrating lymphocytes, tumor growth, and metastasis in a model using a

variety of neutralizing antibodies and gene-targeted mice [58]. RANKL blockade improved the efficacy of anti-CTLA-4 mAbs against solid tumors and experimental metastases. Treg-depleting anti-CTLA-4 mAbs of the mouse IgG2a isotype showed the highest combinatorial activity. The optimal combination depended on the presence of activating Fc receptors and lymphocytes (particularly natural killer and $CD8^+$ T cells), whereas anti-RANKL alone did not require Fc receptors. T-cell infiltration into solid tumors post anti-RANKL and anti-CTLA-4 was significantly higher, and this was accompanied by increased T-cell effector function. Several studies are currently ongoing, studying the effect of denosumab monotherapy and the combination of RANKL inhibition and immunotherapy [2, 7].

3.7 RANK/RANKL Signaling and Chemo- or Radiotherapy

The role of the RANK/RANKL signaling in drug resistance remains unclear. There is some in vitro evidence suggesting that RANK/RANKL signaling can induce chemoresistance through the activation of multiple signal transduction pathways [59, 60]. However, in a mouse model, RANKL blockade increases the efficacy of cisplatin chemotherapy [60]. At the moment, there are no objective data that RANK/RANKL signaling inhibition has an influence on the effectivity of chemotherapy or radiotherapy in humans [7].

3.8 Conclusion

The role of RANK/RANKL inhibition as an immunomodulatory strategy in combination with other treatment modalities should be further investigated. As denosumab has clear immune-stimulating effects and an interesting toxicity profile, the drug has an attractive potential to be coadministered with immunotherapies for cancer treatment, thereby reinforcing the antitumor immune response. Optimal dosage and sequencing of treatment with other drug combinations warrants further investigation.

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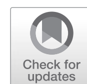

Modulation of Cancer Cell Growth and Progression by Caveolin-1 in the Tumor Microenvironment

4

Hyo-Jin Yoon and Young-Joon Surh

Abstract

Caveolin-1 (Cav-1), a major structural component of cell membrane caveolae, is involved in a variety of intracellular signaling pathways as well as transmembrane transport. Cav-1, as a scaffolding protein, modulates signal transduction associated with cell cycle progression, cellular senescence, cell proliferation and death, lipid homeostasis, etc. Cav-1 is also thought to regulate the expression or activity of oncoproteins, such as Src family kinases, H-Ras, protein kinase C, epidermal growth factor, extracellular signal-regulated kinase, and endothelial nitric oxide synthase. Because of its frequent overexpression or mutation in various tumor tissues and cancer cell lines, Cav-1 has been speculated to play a role as an oncoprotein in cancer development and progression. In contrast, Cav-1 may also function as a tumor suppressor, depending on the type of cancer cells and/or surrounding stromal cells in the tumor microenvironment as well as the stage of tumors.

Keywords

Caveolin-1 · Caveolae · Cancer-associated fibroblasts · Cancer progression · Cancer stem-like cells · Epithelial-mesenchymal transition · Metastasis · Stem cells · Stromal cells · Tumor microenvironment

4.1 Introduction

Caveolae represent a flask-shaped invagination of the plasma membrane that play a role in endocytosis and forming vesicles in the cytoplasm. Caveolae are heterogeneous in normal and tumor cells, and they are most abundant in stromal cells, such as adipocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells [1, 2]. A family of integral membrane proteins, called caveolins, are the principal components of caveolae. Caveolins may act by compartmentalizing and concentrating signaling molecules and are involved in receptor-independent endocytosis [3]. Caveolins have amino-terminal and carboxy-terminal domains

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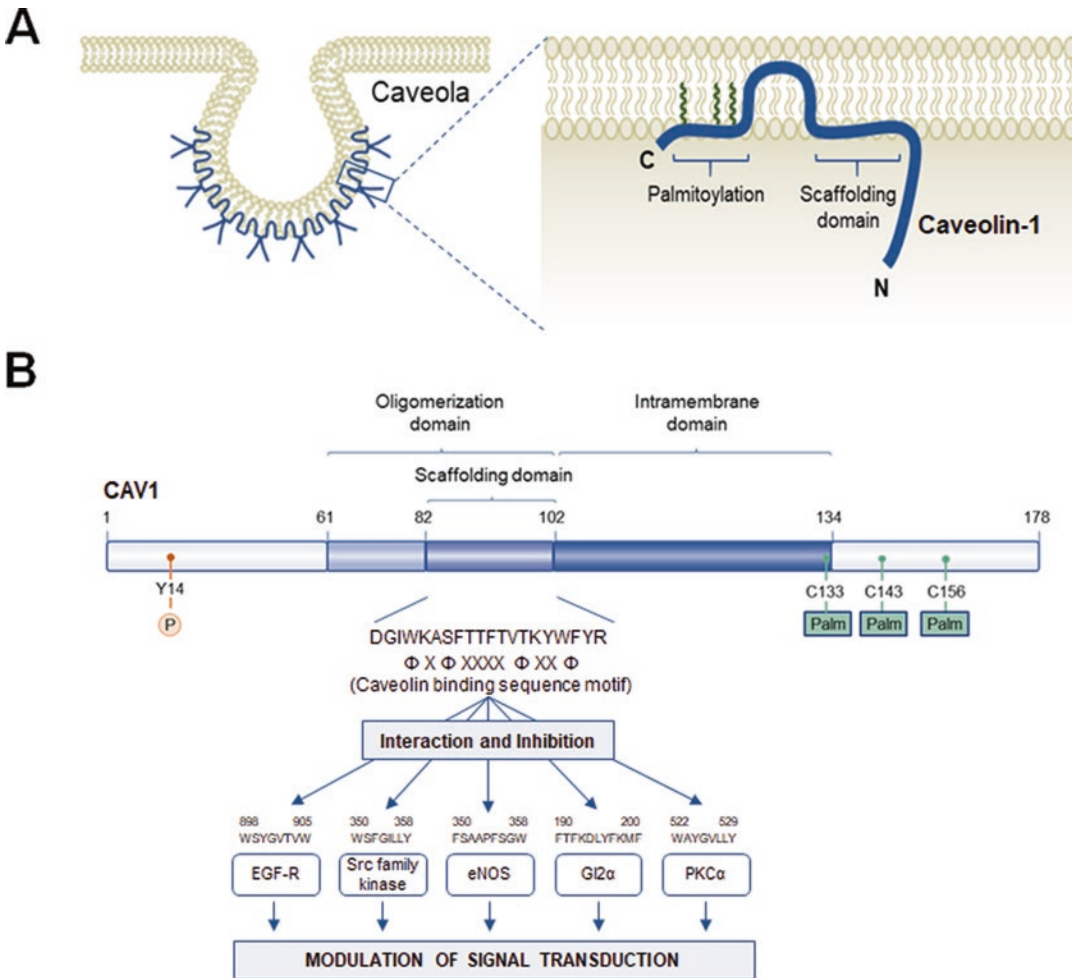

Fig. 4.1 Structures of caveolae and Cav-1. (**a**) The diagrams of caveolae and Cav-1. Cav-1 is inserted into the caveolar membrane, with the N- and C-termini facing the cytoplasm and an intramembrane domain embedded within the membrane bilayer. (**b**) The sequence of the caveolin-scaffolding domain (CSD; residues 82–102) and the caveolin binding sequence motifs within several caveolae-localized signaling molecules are shown. These include epidermal growth factor receptor (EGF-R), Src family tyrosine kinases, endothelial nitric oxide synthase (eNOS), G-protein α subunits (Gi2α), and PKC isoforms (PKCα). In most cases, such interaction is inhibitory, leading to inactivation of the signaling molecules and modulation of downstream signal transduction

localized at the cytoplasmic face of the cell membrane (Fig. 4.1a) [4]. Caveolins also contain the caveolin scaffolding domain required for binding to signaling proteins. Caveolins modulate functions of several signaling molecules, such as Src, G-protein α-subunits, and H-Ras, involved in cell proliferation and growth (Fig. 4.1b) [5]. Caveolins consist of the three core members, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2), and Caveolin-3 (Cav-3). Cav-1 is highly expressed in various cells, such as adipocytes, endothelial cells, fibroblasts, and smooth muscle cells. Cav-2 shares a similar expression profile with Cav-1, as it requires Cav-1 for stabilization. Cav-3 is predominantly expressed in muscle cells [6]. Cav-1 and Cav-3 form homo-oligomers, and oligomerization is essential for caveolae biogenesis. Ablation of Cav-1 and Cav-3 causes a deficiency of caveolae in various cell types [7, 8]. Besides formation of caveolae, caveolins have multiple cellular functions by interacting with signaling molecules, such as receptors, kinases, adhesion

molecules, and G proteins. These include cholesterol homeostasis, vesicle trafficking, and endocytosis [9, 10]. Of three isoforms of caveolin family, Cav-1 is the principal structural component of caveolae, and its expression is essential for driving the formation of morphologically identifiable caveolae [10]. Cav-1, as a scaffolding protein, modulates multiple signal transduction pathways involved in cell cycle progression, cellular senescence, cell proliferation and death, lipid homeostasis, etc.

Over the past years, there has been increasing concern about the involvement of Cav-1 in the development and pathogenesis of human cancer. Cav-1 regulates cancer cell metabolism, proliferation, differentiation, resistance to apoptosis, survival, adhesion, migration, invasion, and metastasis [12–16]. On the other hand, Cav-1 can also act as a tumor suppressor in some circumstances in which its low expression favors tumor progression [17–20]. Besides epithelial Cav-1 in tumors, altered expression of stromal Cav-1 in the tumor microenvironment (TME) is observed in different types of human malignancies [12, 14]. However, the clinical significance of Cav-1 in cancer is still elusive.

This review summarizes the differential roles for Cav-1 in tumor development, migration, metastasis, therapy resistance, and cancer cell survival.

4.2 Cav-1 Expression in Human Cancer

Cav-1 expression has been extensively examined in various tumor specimens from cancer patients as well as in human cancer cell lines [14–18]. In most studies, the association between Cav1 expression levels and clinicopathological significance in terms of prognosis, metastatic status, and/or tumor resistance has been analyzed [14–18]. However, there is a contradictory profile of the Cav-1 expression in human cancer [19]. While some studies suggest the oncogenic function of Cav-1, loss or low expression of Cav-1 has been associated with poor outcomes in various tumor types. In other studies, however, there is no consistent change in Cav-1 expression between cancer cells and their normal adjacent cells. Therefore, the effects of Cav-1 expression on tumorigenicity and aggressiveness appear to vary widely among different cancer types [19].

4.2.1 Oncogenic Function

Cav-1 is frequently overexpressed or mutated in various tumor tissues and cancer cell lines. Aberrant upregulation of Cav-1 has been postulated to favor cancer cell survival and growth. Cav-1 may function as an oncoprotein commonly associated with enhanced malignant behavior, such as metastasis [15, 16, 18] and therapy resistance [14, 15, 17]. The clinicopathologic significance of upregulated Cav-1 is described below.

4.2.1.1 Role in Cancer Cell Invasiveness and Metastasis

In certain tumors, progression into a metastatic or drug-resistant form has been attributable to reexpression of Cav-1 [14–18]. Upregulation of Cav-1 is thought to contribute to cancer cell invasiveness and resistance to anoikis, properties that are essential for metastasis [20]. In non-neoplastic gastric mucosa, Cav-1 was not expressed in the epithelial compartment. However, the expression of Cav-1 was significantly correlated with cancer progression and poor prognosis in gastric cancer. This was associated with an advanced stage and lymph node metastasis [21].

Restoration of Cav-1 expression in lung adenocarcinoma cells is sufficient to promote their filopodia formation, migration, and metastatic potential [22]. Recent studies have indicated that cell invasion during tumor progression may be critically dependent on the acquisition of epithelial-mesenchymal transition (EMT) features. Multiple lines of evidence support that Cav-1 mediate the invasion and metastasis of cancer which are accompanied by EMT. Thus, Cav-1 can promote bladder cancer metastasis by inducing EMT which is linked to activation of phosphatidylinositol 3-kinase-Akt and upregulation of Slug expression [23]. Moreover, overex-

pressed Cav-1 increased vimentin expression, but downregulated E-cadherin. This accompanied the change of EMT, resulting in the increased motility and invasiveness in hepatocellular carcinoma [24]. The reduced levels of Cav-1 in hypoxia stimulate activation of epidermal growth factor receptor and consequently STAT3. This, in turn, results in the downregulation of E-cadherin and upregulation of mesenchymal markers, such as Slug, α-smooth muscle actin, N-cadherin, and vimentin, suggesting that Cav-1 can mediate the EMT and promote invasiveness in gastric cancer [24] (Fig. 4.2).

Matrix metalloproteins (MMPs) are a family of zinc-containing proteolytic enzymes that degrade various components of extracellular matrix [25]. The migration- and invasion-promoting effects of Cav-1 overexpression in hepatocellular carcinoma appear to be mediated by increasing secretion or expression of MMP-2, MMP-9, and MT1-MMP as well as inducing an EMT-like phenotype [26].

Rho-GTPases are involved in tumor metastasis and invasion [27, 28]. Previous studies have indicated the role of Cav-1 in regulating the activity of Rho-GTPases in various metastatic cancers. The interaction between Cav-1 and Rho-GTPases promotes tumor metastasis, which depends on the elevated expression of α5-integrin and the enhanced activation of Src and Ras [29]. The acquisition of the metastatic phenotype requires adhesive interaction between cancer cells and the endothelium, in which focal adhesion kinase (FAK) plays an essential role [30]. The expression of Cav-1 was positively correlated with that of FAK in gastric cancer [21]. Rho/ROCK signaling promotes tumor cell migration and metastasis by regulating focal adhesion dynamics through Cav-1 phosphorylation at the tyrosine 14 residue [31]. Cav-1 tyrosine phosphorylation is dependent on Src kinase and Rho/ROCK signaling. The phosphorylated Cav-1 stabilizes FAK association with focal adhesion and promotes cell migration and invasion [31].

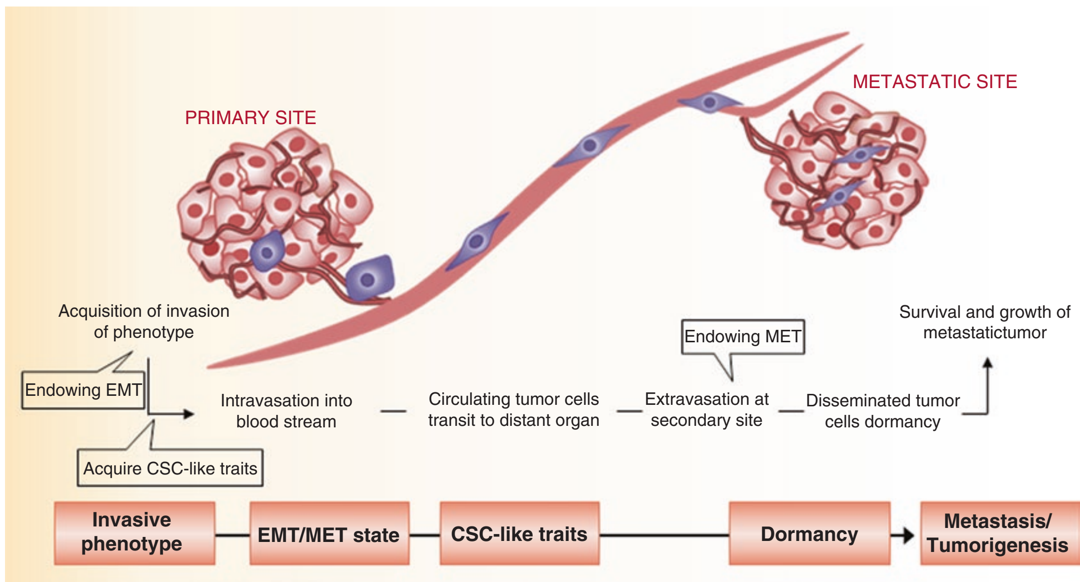

Fig. 4.2 Cancer cells within the primary tumor undergo EMT and acquire stem-like traits (CSCs) and endow invasive capacity, then intravasate into the tumor vasculature in the form of circulating tumor cells (CTCs), which must be able to survive the circulating blood and evade from the innate immune response and other defenses. Once CTCs migrate to a secondary site, the settlement in supportive niches enables them to survive and retain their stem-like tumor-initiating capacity. In the target site, disseminated cancer cells (DTCs) encounter inhibitory signals resulted in the arrested in cell cycle subsequently leading to dormancy from months to decades while they adapt to their new found microenvironment. Cancer cells undergo mesenchymal-to-epithelial transition (MET) in order to acquire feature proliferation to metastatic outgrowth in the target site

4.2.1.2 Role in Therapy Resistance

Increased expression of Cav-1 can promote development of resistance to chemo- or radiotherapy [14, 17]. In cisplatin-resistant ovarian cancer cells, both expression levels of Cav-1 and its mRNA transcript were significantly higher than those in normal ovarian cancer cells [32]. Knockdown of Cav-1 sensitized cisplatin-resistant ovarian cancer cells to apoptosis, which was attributable to downregulation of expression of Notch-1, p-Akt, and p-NF-κB/p65 [32].

4.2.1.3 Role in Cancer Stem Cells (CSCs)

CSCs represent an important subset of TME components. CSCs are responsible for tumor initiation, metastasis, and recurrence as well as resistance to chemo- and radiotherapy, which are associated with poor clinical outcomes. Because CSCs contribute to cancer development and progression, the presence of CSC population in precancerous stage is an early indicator of malignant progression. Some biological mediators (e.g., nitric oxide; NO) found in the TME could promote manifestation of CSC-like phenotypes of human nonsmall-cell lung carcinoma via Cav-1 upregulation [33].

Our recent study revealed that Cav-1 expression is significantly lower in tumorspheres derived from human breast cancer (MDA-MB-231) cells than in adherent cells [34]. In line with this notion, silencing of Cav-1 enhanced stemness of MDA-MB-231 cells as evidenced by the increased proportion of $CD44^{high}$ and $CD24^{low}$ cells. Notably, Src-mediated phosphorylation of Cav-1 at the Tyr 14 residue was found to be essential for its destabilization via the ubiquitin-proteasome degradation system which accounts for the reduced Cav-1 in a breast CSC-like state [34].

4.2.2 Tumor Suppressive Function

In some tissues, Cav-1 has been shown or speculated to function as a tumor suppressor. Several studies have shown that Cav-1 inhibits colony formation and induces apoptosis in transformed cells and cancerous cells [35–37]. In addition, forced reexpression of Cav-1 abrogated anchorage-independent growth of transformed cells [35–39].

In human breast cancer, the *Cav-1* has been considered a tumor suppressor gene associated with inhibition of tumor metastasis. Sagara and colleagues investigated the mRNA and protein expression levels of Cav-1 in 162 cases of breast cancer and found that the Cav-1 expression was suppressed at both transcriptional and translational levels in breast cancer tissues compared with the normal tissues [40]. In this study, the reduced *Cav-1* mRNA level was significantly associated with an increased tumor size, and was correlated with hormonal receptor status [40]. Overexpressed Cav-1 reduced the invasion capacity of metastatic mammary tumor cells by inhibiting the activity of MMP-2 and MMP-9 [41]. In normal breast, Cav-1 was found to be expressed in myoepithelial cells, endothelial cells, and a subset of fibroblasts. In contrast, luminal epithelial cells showed negligible staining [42].

Low levels of Cav-1 and its mRNA transcripts were detected in several colon carcinoma cell lines. Moreover, Cav-1 protein levels were markedly lower in human colon tumor epithelium than in normal colon mucosa. Ectopic expression of Cav-1 in the colon carcinoma cells attenuated tumor formation when these cells were inoculated into nude mice [43]. Moreover, Cav-1 may function as a negative regulator of metastasis by inhibiting MT4-MMP expression in colon cancer [44]. Cav-1 is not expressed in lipid rafts of the highly metastatic colon cancer cell line, but expressed in cytosolic fractions of the parental lower metastatic cell line. Xenografting Cav-1 deficient cells in nude mice induced development of bigger tumors expressing higher levels of proliferating cell nuclear antigen than in mice injected with cells expressing the higher level of Cav-1 [45]. In another study, high Cav-1 expression correlated with good clinical outcomes in head and neck cancer and extrahepatic biliary carcinoma cells [46]. In mucoepidermoid carcinoma of the salivary glands, reduced expression of Cav-1 was associated with a poor prognosis for some patients [45].

Contrary to the previous report on the association between Cav-1 and Rho-GTPases that promotes tumor metastasis [29], Lin and colleague have reported that Cav-1 expression inhibits RhoC GTPase activation and subsequently activates the p38 mitogen-activated protein kinase, leading to suppression of migration and invasion of primary pancreatic cancer cells [47].

In in vivo experiments, Cav-1 knockout mice showed increased development or progression of some cancers. A novel mouse model of colorectal cancer was generated by crossing C57BL/6 *Apc*$^{min/+}$ with B6129 Cav-1 knockout (*Cav1*$^{-/-}$) mice. Absence of Cav-1 accelerated colorectal tumorigenesis in *Apc*$^{min/+}$ mice, which was accompanied by upregulation of Wnt signaling [48].

Cav-1 null mice are much more susceptible to chemically induced skin carcinogenesis as well as epidermal hyperplasia than wild-type littermates [49]. In addition, cyclin D1 expression was upregulated during epidermal hyperplasia, which may account for the increased susceptibility of Cav-1 null mice to skin paillomagenesis [49]. Further, orthotopic implantation of B16F10 melanoma cells in the skin of Cav-1 null mice increased tumor growth [50].

Lewis lung carcinoma cells implanted into Cav-1 knockout mice had increased tumor vascular permeability compared with tumors implanted into wild-type mice. Cav-1 deficient mice also had significantly higher tumor growth rates, and this was attributable to increased tumor angiogenesis and decreased tumor cell death [51].

4.3 Stromal Versus Tumoral Cav-1 in TME

There has been increasing concern about the tumor-host interactions, which influence tumor growth, metastasis, therapy resistance, and cell survival. Understanding such tumor-stroma communication interactions may hence offer a novel therapeutic strategy to avoid or minimize therapy resistance and improve clinical outcomes [14].

Multiple lines of compelling evidence support that the heterogeneous tumor stroma in TME contributes to manifestation of a malignant phenotype of epithelial tumors, tumor recurrence, metastasis, and therapy resistance, resulting in poor clinical outcome. In this context, Cav-1 in the stroma of TME is also likely to be an important prognostic indicator of breast cancer [12, 14]. An absence or reduced stromal Cav-1 expression accounts for poor clinical outcome or therapy resistance in many different types of cancers [12, 13, 52–54].

4.3.1 Cav-1 in Cancer-Associated Fibroblasts

The stroma which constitutes at least half of the tumor mass consists of cancer-associated fibroblasts (CAFs), macrophages and other immune cells, and endothelial cells. Of the stromal cells, CAFs play a key role in tumor-stromal interaction. Loss of Cav-1 expression in CAFs results in an activated TME, thereby driving early tumor recurrence, metastasis, and poor clinical outcome in various malignancies [12]. The loss of Cav-1 in fibroblasts is sufficient to induce a CAF phenotype. In addition to CAFs, metastasis-associated macrophages in TME also express abundant levels of Cav-1, which is critical for metastasis and not for primary tumor growth [55]. The decreased expression of Cav-1 in CAFs resulted in a growth advantage and the chemoresistance of cancer cells when they were co-injected into immunodeficient mice to develop mixed fibroblast/cancer cell xenografts [56]. In this study, however, Cav-1 downregulation in cancer cells had no effect on chemoresistance and growth gain in vivo. Thus, it is likely that relative expression of tumor vs. stromal Cav-1 in TME has more precise prognostic significance than that of each alone. In this context, it is interesting to note that low expression of stromal Cav-1 was negatively associated with cytoplasmic Cav-1 expression in total tumor tissues [57]. As the colon tumor becomes more aggressive and metastatic, it looses the stromal Cav-1 and gains the cellular Cav-1 as well as the abnormal β-catenin expression [58]. In line with this notion, the high tumor/low stromal expression of Cav-1 was closely associated with poor

prognostic outcomes in primary human prostate cancer patients [59].

Sotgia and colleagues have proposed paracrine signaling mechanisms by which the loss of stromal Cav-1 promotes tumor progression to fuel the growth of adjacent tumor cells [12]. It appears that oxidative stress is the root cause of initiation of the loss of stromal Cav-1 via autophagy [12]. It is noteworthy that loss of stromal Cav-1 correlates with high epithelial Cav-1 levels and activated Akt [60]. Low stromal expression of Cav-1 increased TGF-β1 expression and induced phosphorylation and activation of Akt in human dermal fibroblasts [61].

Though the majority of studies suggest stromal Cav-1, especially of CAF origin, has tumor suppressive functions, Cav-1 expression of CAFs has been shown to be associated with patients' poor prognosis [62, 63]. Moreover, tumors with Cav-1-positive CAFs had vascular and pleural invasion significantly more frequently than those with Cav-1-negative CAF [64].

4.3.2 Cav-1 in Other Stromal Cells

Besides CAFs, Cav-1 may also functions in some other stromal cells in TME. Cav-1 is abundant in endothelial cells, adipocytes, and smooth muscle cells as well as in fibroblasts and epithelial cells. Several studies have suggested that Cav-1 may function in the main types of vascular cells in TME [51, 65–67], including pericytes, endothelial cells, and smooth muscle cells which are associated with vascular permeability and morphogenesis in tumor. Endothelial cells play a central role in angiogenesis, a process by which new vasculature is derived from preexisting blood vessels. Several studies have proposed a role for Cav-1 in the regulation of vascular development and angiogenesis [51, 65–67].

Under physiological condition, the main function of Cav-1 is to inhibit endothelial permeability. Cav-1 knockout mice were observed to exhibit a hyperpermeable vascular endothelium [66]. Likewise, tumors grown in *Cav1*$^{-/-}$ mice became leaky as evidenced by increased tumor vascular permeability, and grew faster, compared with tumors implanted into wild-type mice [51]. Cav-1 deficient mice also displayed elevated tumor angiogenesis and decreased tumor cell death, which may account for significantly higher tumor growth rates [51]. As Cav-1 is an endogenous inhibitor of endothelial NO synthase (eNOS), the loss of Cav-1 may result in hyperactivation of eNOS, and resultant NO overproduction is speculated to increase tumor vascular permeability, survival, and ultimately tumor growth [51]. Besides inhibition of endothelial NO production, there might be an alternative mechanism by which Cav-1 modulates the microvascular permeability and angiogenesis. Cav-1 has been found to interact with many intracellular signaling molecules including receptors, thereby altering their activity. For instance, Cav-1 suppresses vascular endothelial cell growth factor receptor (VEGFR)-2 signaling by inhibiting tyrosine phosphorylation of this receptor mediated by adherens junction protein, VE cadherin [51]. Therefore, the enhanced tumor permeability and growth as a consequence of loss of Cav-1 may be attributed to augmented proangiogenic signaling through inhibition of phosphorylation-dependent VEGFR-2 activation [51].

Soon after microvessels are formed, they come in close contact with mural cells of the smooth muscle cell lineage, referred to as pericytes or vascular smooth muscle cells. Such association of pericites (smooth muscle cells) with endothelial cells lining newly formed blood vessels is essential for vascular development and stability [68]. Cav-1 was found to be enriched in the lipid raft fraction of pericytes [69]. Cav-1 impaired the migration of pericytes [66]. Therefore, a decrease in Cav-1 abundance appears to stimulate the angiogenesis and prevent its termination by mural cell recruitment [66]. In another study, a cell-permeable peptide derived from the Cav-1 scaffolding domain inhibited the proliferation of pericytes, but not their survival or migration [67].

There is paucity of information on the role of Cav-1, derived from other stromal cells of TME, in cancer development and progression. Cav-1 promotes differentiation of monocytes to macrophages [70]. Downregulated Cav-1 expres-

sion in circulating monocytes has been implicated in the pathogenesis of psoriasis [71]. However, the functional role of Cav-1 in stromal macrophages in TME has been poorly understood. It has been reported that Cav-1 functions as an anti-metastatic regulator in mouse models of lung and breast cancer pulmonary metastasis [54]. Among all the recruited inflammatory cell populations, metastasis-associated macrophages (MAMs) uniquely express high levels of Cav-1. Loss of Cav-1 did not affect MAM recruitment to the metastatic site, but rather favored lung metastatic growth through increased angiogenesis [54].

4.4 Role of Cav-1 in the Cancer Cell Metabolism and Metabolic Reprogramming of the Tumor Stroma

Recent studies have highlighted the importance of Cav-1, especially of stromal origin, in metabolic alterations in cancer cells in relation to their survival advantage. Cav-1 influences tumor development or progression by modulating such metabolic pathways as glycolysis, mitochondrial bioenergetics, glutaminolysis, fatty acid metabolism, etc. [13]. Catabolic CAFs represent a key metabolic "fuel source," required for cancer cell propagation, survival, and systemic dissemination during metastasis [52]. A loss of Cav-1 has been shown to drive the metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells. Stromal cells could function as providers of energy metabolites for tumor cells by undergoing the "reverse Warburg effect" [53]. The interaction between the tumoral microvesicles (TMVs) and stroma in the tumor microenvironment plays a critical role in facilitating cancer progression. After being incubated with tumoral microvesicles, normal human gingival fibroblasts acquired a phenotype switch to CAFs which was accompanied by degradation of Cav-1 [72]. Notably, Cav-1-deficient CAFs undergo autophagy to secrete energy-rich metabolites and chemical building blocks that can sustain and support the growth of tumor cells [12].

Some studies also revealed the critical role of oxidative stress in a loss of stromal Cav-1 and the metabolic reprogramming of CAFs [73]. Although Cav-1 loss is caused by elevated ROS levels, Cav-1 downregulation may result in increased oxidative stress, which represents a feed-forward mechanism [12]. Oncogenes drive the onset of the CAF phenotype in adjacent normal fibroblasts by provoking oxidative stress. This oncogene-triggered fibroblast activation is "mirrored" by a loss of stromal Cav-1. These fibroblasts exhibit elevated ROS production and elevated glucose uptake, indicative of a shift toward a glycolytic metabolism [52].

4.5 Conclusion

As a main component of caveolae, Cav-1 is involved in many biological processes that include substance uptake and transmembrane signaling. In addition, Cav-1 can modulate cancer cell proliferation, differentiation, migration, invasion, metastasis, and resistance to anticancer therapy.

Although the role of Cav-1 in cancer is still elusive, the majority of reports suggest that Cav-1 represents an important prognostic marker of tumor development and progression, and independently serves as a predictor of overall survival rate. In addition, through interaction with other biological molecules, Cav-1 modulates stem-like traits. On the other hand, a functional loss of Cav-1 in several tumor cells induces a hyperproliferative state, promoting cell proliferation, survival, and invasiveness as well as acquisition of resistance to cancer therapy [14].

Based on these findings, the roles for Cav-1 in human cancer and its suitability as a prognostic marker are controversial. Cav-1 is likely to function both as a tumor suppressor and as an oncoprotein, depending on the stage of neoplastic transformation and extent of tumor progression (Fig. 4.3). Though Cav-1 appears to be downregulated in early transformed cells, a reexpression or rather upregulation and stabilization through

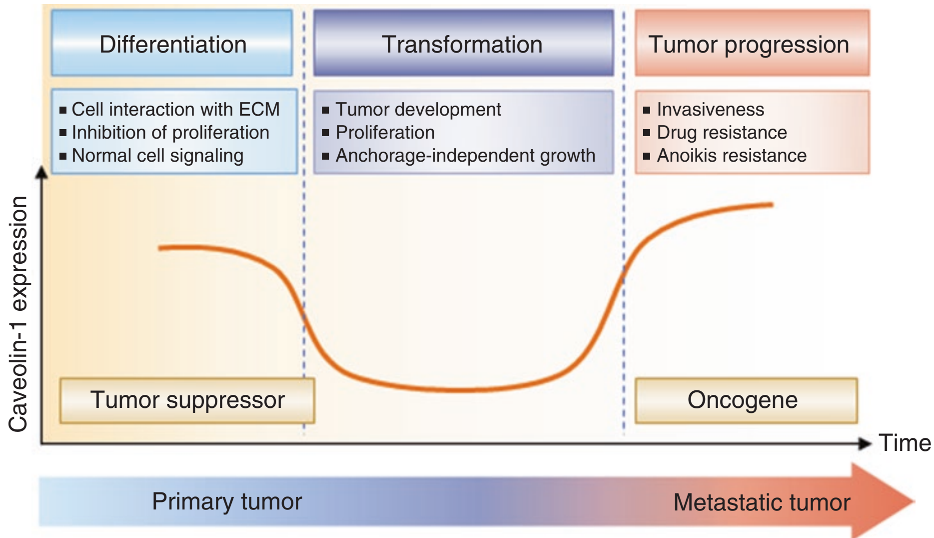

Fig. 4.3 Dual role of Cav-1 in cancer. Cav-1 may function both as a tumor suppressor and as an oncoprotein, depending on the stage of oncogenic transformation and extent of tumor progression. Cav-1 is expressed at relatively high levels in many differentiated cells. During oncogenic transformation, Cav-1 is downregulated, in certain tumors, further progression into a metastatic or drug-resistant form is associated with reexpression of Cav-1. Upregulation of Cav-1 in these tumors is thought to contribute to tumor cell invasiveness and resistance to anoikis, properties that are essential for tumor cell metastasis. Increased Cav-1 has also been associated with the development of drug resistance in tumors

phosphorylation of Cav-1, in later tumor stages, may confer invasiveness, resistance, and survival advantage of multidrug-resistant tumor cells [14].

The differential effects of Cav-1 in tumor development and progression may be related to a different profile of TME components involved in each stage of cancer, particularly in the context of tumor vs. stromal form of Cav-1. Loss of stromal Cav-1 in TME has been frequently associated with poor patient outcomes in diverse malignancies. A characteristic shift in stromal-epithelial Cav-1 in advanced and metastatic tumor stages with a loss of stromal Cav-1 and a concomitant increase in expression of epithelial isoform highlights Cav-1 as being a tissue and stage-specific tumor modulator [14]. A molecular mechanism by which Cav-1 expression is upregulated/restored in more advanced stages of cancer and how Cav-1 deficient CAFs promote this process merit further investigation. Identification and characterization of CAF-derived signaling molecules that mediate the shift in stromal-tumor Cav-1 accumulation during cancer progression will be of particular interest. Another interesting research subject would be elucidation of how metabolic reprogramming of Cav-1 deficient CAFs by CAF-addicted cancer cells is achieved.

Further studies are required to unveil the clinical value of Cav-1 as a prognostic marker and a candidate target for cancer therapy.

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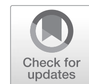

5 Exosomes: Novel Players of Therapy Resistance in Neuroblastoma

Heather Richard, Arya Pokhrel, Srinivas Chava, Anup Pathania, Santharam S. Katta, and Kishore B. Challagundla

Abstract

Neuroblastoma is a solid tumor (a lump or mass), often found in the small glands on top of the kidneys, and most commonly affects infants and young children. Among neuroblastomas, high-risk neuroblastomas are very aggressive and resistant to most kinds of intensive treatment. Immunotherapy, which uses the immune system to fight against cancer, has shown great promise in treating many types of cancer. However, high-risk neuroblastoma is often resistant to this approach as well. Recent studies revealed that small vesicles known as exosomes, which are envelopes, could deliver a cargo of small RNA molecules and provide communication between neuroblastoma cells and the surrounding cells and trigger metastasis and resistance to immunotherapy. In this chapter, we describe the role of exosomes and small RNA molecules in the metastasis and regression of neuroblastoma and the potential therapeutic approaches to combat this menace.

Keywords

Neuroblastoma · Exosomes · Non-coding RNAs · MYCN · Metastasis · Extracellular vesicles · Multivesicular bodies · Exosome biogenesis · Tumor microenvironment · Tumor-associated monocytes · Pericytes · Disialoganglioside · Antibody-dependent cell cytotoxicity · Chemotherapy · Immunotherapy

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Abbreviations

3′-UTR	Three prime untranslated region
ADCC	Antibody-dependent cell cytotoxicity
AURKA	Aurora kinase A
EFS	Event-free survival
ESCRT	Endosomal-sorting complex required for transport
GD2	Disialoganglioside
IL-15	Interleukin-15
IL-2	Interleukin-2
ILV	Intraluminal vesicle
MAb	Monoclonal antibody
miRNA	MicroRNA
mRNA	Messenger RNA
MSCs	Mesenchymal stem/stromal cells
MVBs	Multivesicular bodies
MYCN	v-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived
NEDD4	Neuronal precursor cell-expressed developmentally downregulated 4
NF-κB	Nuclear factor-kappa B
NK	Natural killer
PCR	Polymerase chain reaction
PNTs	Peripheral neuroblastic tumors
RNA	Ribonucleic acid
TERF1	Telomeric repeat-binding factor 1
TGFβ 1	Transforming growth factor beta 1
TGFβR1	Transforming growth factor beta receptor 1
TGFβR2	Transforming growth factor beta receptor 2
TLR8	Toll-like receptor 8

5.1 Neuroblastoma

Neuroblastoma is an embryonal tumor of the autonomic nervous system. This means that the origin of a cell is preceded to be developing immaturely in the neural-crest tissues [26, 29]. Neuroblastoma is the most common solid tumor found in infants and children. They account for almost 8–10% of all childhood tumors. The median age of diagnosis with neuroblastoma is 17 months [22]. Almost 15% of all deaths that are related to this cancer are in pediatrics [26, 29]. Almost 500 new cases are reported every year [26, 29]. 90% of cases are usually diagnosed before the age of five, and 30% of those are within the first year of life [11]. Neuroblastoma has been found to be more prevalent in males compared to females [26, 29], and the occurrence of neuroblastoma is unusual in adolescents and adults. 95% of all neuroblastomas occur in children under five years of age [11]. However, cases have been detected pre-birth, during an ultrasound examination. Many patients diagnosed with neuroblastoma have shown to undergo immense relapse of neuroblastoma. In infants, the prognosis is very good, while it is somewhat at a disadvantage in older children. The patient outcome with the diagnosis of neuroblastoma has improved over the last 30 years. The 5-year survival rates in low- and medium-risk patients vary from 52% to 74% [26]. There is a prediction that around 50–60% of patients diagnosed with high-risk neuroblastoma will relapse [26]. The tumors begin in tissues of the sympathetic nervous system. This may cause a mass in the neck, chest, abdomen, or pelvis. A mass can either cause no symptoms or may progress into a tumor that causes severe illness. The diagnosis of neuroblastoma is 10.2 cases per million children under 15 years of age, and it is the most common cancer diagnosed during the first year of life [21, 26].

5.2 Exosomes

Exosomes are small extracellular vesicles secreted from cells and have lately attracted the attention of researchers worldwide owing to their critical role in intercellular signaling and disease. These are nanoparticles ranging from approximately 30–100 nm in size [41]. Exosomes are released out of the cell into the extracellular surrounding after multivesicular bodies (MVBs) fuse with the cellular membrane. All biological fluids tested have been shown to contain vesicles, including in vitro grown cell lines, which have also been shown to release vesicles to different extents. Canonical exosomes display a particular

biconcave or cup-like shape when produced by artificially drying during preparation, while they appear spheroid in solution under a transmission electron microscope [31]. Typically, they have a density range from 1.13 g/mL (B cell-derived exosomes) [31] up to 1.19 g/mL (epithelial cell-derived exosomes) [31] on sucrose gradients.

Exosome biogenesis begins within the endosomal system. The early endosomes grow into multivesicular bodies (MVBs). During this process, the endosomal membrane encloses to generate intraluminal vesicles (ILVs) in the lumen of the organelles [15, 21]. The protein sorting of ILVs is a highly regulated mechanism that is dependent on the endosomal sorting complex required for transport (ESCRT) machinery [24] or ESCRT-independent mechanism [16]. Both pathways are not entirely separated. ESCRT has four different protein complexes: ESCRT-0, ESCRT-1, ESCRT-2, and ESCRT-3 [32].

The ESCRT mechanism is initiated by recognition and sequestration of ubiquitinated proteins to specific domains of the endosomal membrane via ubiquitin binding subunits of ESCRT-0. After interaction with ESCRT-I and -II complexes, the total complex will then combine with ESCRT-III, a protein complex that is involved in promoting the budding processes. Finally, following cleaving the buds to form ILVs, the ESCRT-III complex separates from the MVB membrane with energy supplied by the sorting protein Vps4 [32]. Despite the controversy of whether exosome release is an ESCRT-regulated mechanism, different ESCRT components and ubiquitinated proteins have already been identified in exosomes isolated from various cell types. Additionally, the typical exosomal protein Alix, which is associated with several ESCRT (TSG101 and CHMP4) proteins, has been reported to participate in endosomal membrane budding and abscission, as well as exosomal cargo selection via interaction with syntenin [32]. These observations led to a hypothesis implicating ESCRT function in exosomal biogenesis. ESCRT-independent manner depends on raft-based microdomains for the lateral segregation of cargo within the endosomal membrane. These microdomains are thought to be highly enriched in sphingomyelinases, from which ceramides can be formed by hydrolytic removal of the phosphocholine moiety [32]. Ceramides are known to induce lateral phase separation and coalescence of microdomains in model membranes. Moreover, the cone-shaped structure of ceramide might cause a spontaneous negative curvature of the endosomal membrane, thereby promoting domain-induced budding.

Consequently, this ceramide-dependent mechanism emphasizes the key role of exosomal lipids in exosome biogenesis [32]. Proteins, such as tetraspanins, also participate in exosome biogenesis and protein loading. Tetraspanin-enriched microdomains (TEMs) are ubiquitous specialized membrane platforms for compartmentalization of receptors and signaling proteins in the plasma membrane [32]. It has been shown that TEMs, together with tetraspanin CD81, plays a key role in sorting target receptors and intracellular components toward exosomes [32]. Exosomes play a critical role in physiological and pathological settings, strategies that interfere with the release of exosomes; and the impairment of exosome-mediated cell-to-cell communication could potentially be used in the future [32]. The general structure of the exosome molecule is given in Fig. 5.1.

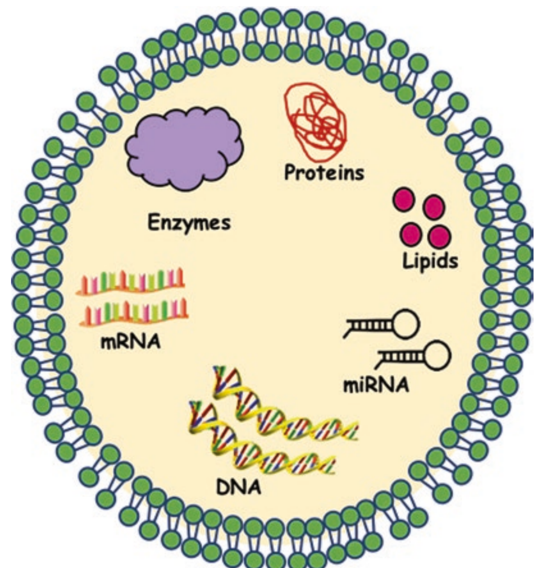

Fig. 5.1 General structure of exosome molecule

5.3 Neuroblastoma and Exosomes

The majority of neuroblastoma deaths occur within two years of diagnosis due to the aggressiveness of the cancer. Exosomes are released by many cell types and transfer their molecular cargo to the target cells, thereby modulating the signaling pathways in the recipient cells. Fibroblasts, endothelial cells, and infiltrating immune cells are the major cell types within a tumor microenvironment that interacts with tumor cells by exosome signaling [7]. The consequences of these interactions depend on the origin of the exosomes determining the exosome cargo. Stressful conditions such as hypoxia, starvation, and acidosis increase exosome release from malignant cells leading to tumor microenvironment alteration and expansion, which subsequently results in tumor progression. Several pieces of evidence show that the major mechanism involved in tumor progression is the role of tumor-derived exosomes in cellular communication. Cancer cell-derived exosomes operate numerous functions. These include angiogenesis, anti-tumor immune responses, and metastatic ability, whereas the non-cancer cell-derived exosomes function by promoting the inhibition of malignant cells [5, 25, 27]. A transmission electron microscopy image of the exosome, showing 95-nm size, enclosed by a lipid bilayer, isolated from neuroblastoma (CHLA-255) cell culture supernatant is given in Fig. 5.2.

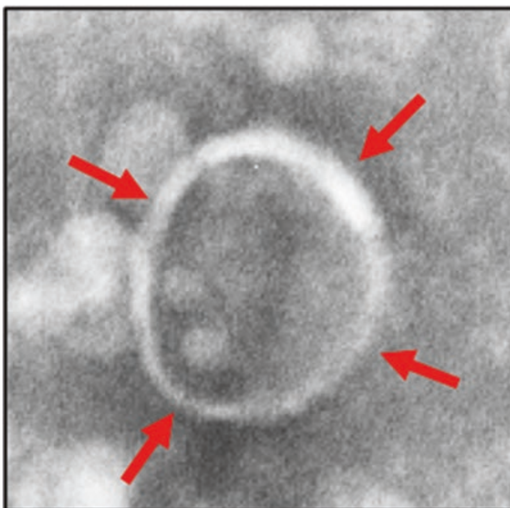

Fig. 5.2 A transmission electron microscopy image of the exosome, showing 95-nm size, enclosed by a lipid bilayer, isolated from neuroblastoma (CHLA-255) cell culture supernatant. Scale: 100 nm

Nearly 20–30% of neuroblastoma cases are associated with the amplification of N-Myc oncogene, which are considered as high risk. Even though it is known that exosomes secreted from N-Myc-amplified neuroblastoma cells contain a tumor-specific signature, it is not known whether exosomes derived from N-Myc-amplified neuroblastoma cells can transfer the aggressive phenotype including chemoresistance between the cells. To test this, exosomes were isolated from derived N-Myc-amplified cancer cells and added to the non-Myc-amplified cell culture, and their properties studied. Addition of exosomes to the non-N-Myc-amplified cells induced migration, colony-forming abilities, and protected the cells against doxorubicin-induced apoptosis. This suggests that exosomes derived from N-Myc-amplified cancer cells can transfer the aggressive phenotype to the neighboring cells, thereby aiding in cancer progression. Proteomic analysis of N-Myc-amplified cancer cell exosomes showed enrichment of TGS101, FlOT1, and VPS35. In addition, exosomes of N-Myc-amplified cells are also enriched in signaling proteins such as NEDD4, β-catenin, and RhoA [10, 14].

In addition to proteins, exosomes carry various molecules, including mRNAs, DNA, and microRNAs (miRNAs). MiRNAs play a significant role in the regulation of genes. MiRNAs work by inhibiting the translation of messenger-RNAs (mRNAs) or inducing mRNA breakdown by binding to the 3′-untranslated region (UTR) of the mRNAs [6]. The primary function of the miRNAs is the downregulation of gene expression. Recent studies on NB cell line exosomes explored the several miRNAs in them, and functional studies of these miRNAs revealed their profound influence on the target cells. Challagundla et al. purified exosomes from neuroblastoma cell lines (SK-N-B(E)2, CHLA-255, and IMR-32) and quantified the content of miR-

21, miR-29a, and miR-155 by quantitative real-time PCR. Among these miRs, miR-21 and miR-29a are implicated in inflammatory reactions in lung cancer; miR-155 is induced during macrophage inflammatory response. In NBL cell line exosomes, miR-21P has been shown to be the top represented miRNA [7]. Monocytes on co-culture with the neuroblastoma cell lines revealed that miR-21 is transferred to human monocytes through exosomes. In monocytes, miR-21 induces upregulation of miR-155 levels in a Toll-like receptor-8 (TLR8)-dependent manner, and miR-155 is transferred from monocytes to neuroblastoma cells through exocytic vesicles. Exosomal targeting of miR-155 in NBL cell lines leads to the downregulation of TERF1 mRNA, an inhibitor of telomerase, thereby leading to increased telomerase activity. Higher telomerase activity is commonly associated with chemotherapy resistance in neuroblastoma patients through a novel exosomic miR-21/TLR8-NF-κB/miR-155/TERF1 signaling pathway [7]. The functional transfer of exosomal miRNAs from neuroblastoma cell to the surrounding monocyte and the development of chemotherapy resistance are given in Fig. 5.3.

Pericytes are a type of fibroblast-like cells, capable of tumor homing and constitute one of the main components of the tumor microenvironment. Pericytes were first named as adventitial cells by Rouget in the nineteenth century and named as pericyte by Zimmermann in 1923. Pericytes are located within the basement membrane of the on-blood vessel walls, thus regulating blood flow, blood vessel permeability, and stabilization of the vascular wall. However, how many types of pericytes present and their role on the development of angiogenesis are not known until Birbrair et al. discovered a mechanistic approach in 2014 using a series of in vitro and in vivo experimentation involving a double transgenic Nestin-GFP/NG2-DsRed mice [4]. The authors identified two pericyte populations: type 1 pericytes expressing Nestin-GFP(-)/NG2-DsRed(+)] and type 2 pericytes expressing Nestin-GFP(+)/NG2-DsRed(+). These pericyte populations were functionally characterized using several in vitro assays and confirmed that type 2 pericytes, but not type 1, exhibit angio-

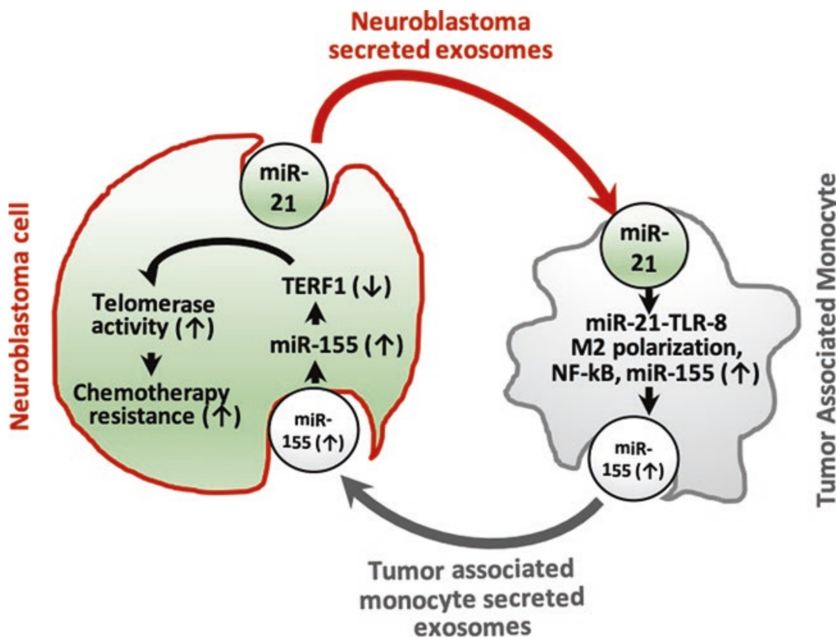

Fig. 5.3 A model depicting the transfer of exosomal miRNAs from neuroblastoma cell to the surrounding monocyte and the development of chemotherapy resistance

genic potential and were recruited during tumor angiogenesis, raising more questions on the potential role of exosomes secreted from pericytes within the tumor microenvironment [4].

In neuroblastoma, cancer cells modulate the tumor microenvironment and play a role in immune escape mechanisms and drug resistance. Neviani et al. have shown that interleukin-15 (IL-15)-activated natural killer (NK) cells secrete exosomes, which exhibit cytotoxicity against MYCN-amplified neuroblastoma cells. The cytotoxic potential of these exosomes was partly dependent upon the expression of miR-186. Interestingly, authors have shown that miR-186 has been shown to be downregulated in high-risk neuroblastoma patients, and its lower levels are a poor prognostic factor. MiR-186 ectopic expression has been shown to downregulate the expression of neuroblastoma oncogenes – MYCN, AURKA, TGFβR1, and TGFβR2. In addition, ectopic expression of miR-186 in MYCN-amplified neuroblastoma cell lines inhibits its growth and migration. Targeted delivery of miR-186 to MYCN-amplified neuroblastoma or NK cells resulted in inhibition of neuroblastoma tumorigenic potential and prevented the TGFβ1-dependent inhibition of NK cells. Neviani et al. have shown targeted delivery of miR-186 in high-risk neuroblastoma is practicable and may lead to an inhibition of tumor growth and spreading [28]. The discovery that NK exosomes are cytotoxic and have their own killing ability even in an immunosuppressive microenvironment supports the belief of including ex vivo derived NK exosomes as a potential future benefit alongside the NK cell-based immunotherapy [28]. A schematic model of releasing exosomes into extracellular space is given in Fig. 5.4.

Most of the studies on exosomes' role in neuroblastoma are based on cell culture experiments, but studies on human neuroblastoma patient exosomes were lacking. To investigate the functions of tumor-derived exosomal miRNAs in neuroblastoma patients in progression and migration of neuroblastoma cells, Ma et al. utilized plasma-derived exosomes and carried out differential exosomal miRNA expression profiles [23]. Ma et al. identified that the expression of hsa-miR199a-3p is significantly upregulated, and strongly correlates with the severity in neuroblastoma patients. Exosomal hsa-miR199a-3p promotes tumor proliferation and migration via decreasing neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) expression in neuroblastoma. Ma et al. have shown that hsa-miR199a-3p may inhibit NEDD4 expression by binding to the 366–373 site of the 3′-UTR of NEDD4 mRNA in neuroblastoma cells, thereby miR-199a-3p promotes proliferation and facilitates migration of NB cells by regulating NEDD4 expression [23]. This work has shown that exosomal hsa-miR-199a-3p can be utilized as a fast, easy, and non-invasive detection biomarker and contribute to the development of novel therapeutic strategies for neuroblastoma in the future. Thus, the content analysis of the exosomes reveals their function in tumor microenvironment progression in malignancies, and this will further lead to developing more efficient micro vesicle-based strategies for cancer prognosis and therapy.

5.4 Treatment Options for Neuroblastoma

Treatment for neuroblastoma depends on the classification of the tumor. There are three broad categories: Low-risk, intermediate-risk, and high-risk [7–9]. Low-risk patients include those with localized tumors and tumors that show characteristics that indicate the tumor is not likely to come back. Low-risk patients are subject to minimal treatment or none at all [9]. Surgery may be the best option for these patients if the tumor is small enough to remove easily. Chemotherapy may be used as a treatment post-surgery, but most often, the patients are monitored for recurrence [9]. Chemotherapy used in low-risk patients includes a mixture of carboplatin, cyclophosphamide, doxorubicin, and etoposide most often [9]. Infants with very small tumors are usually monitored because these tumors are likely to disappear on their own without treatment [9].

Patients are classified as having intermediate risk if the tumor shows different characteristics,

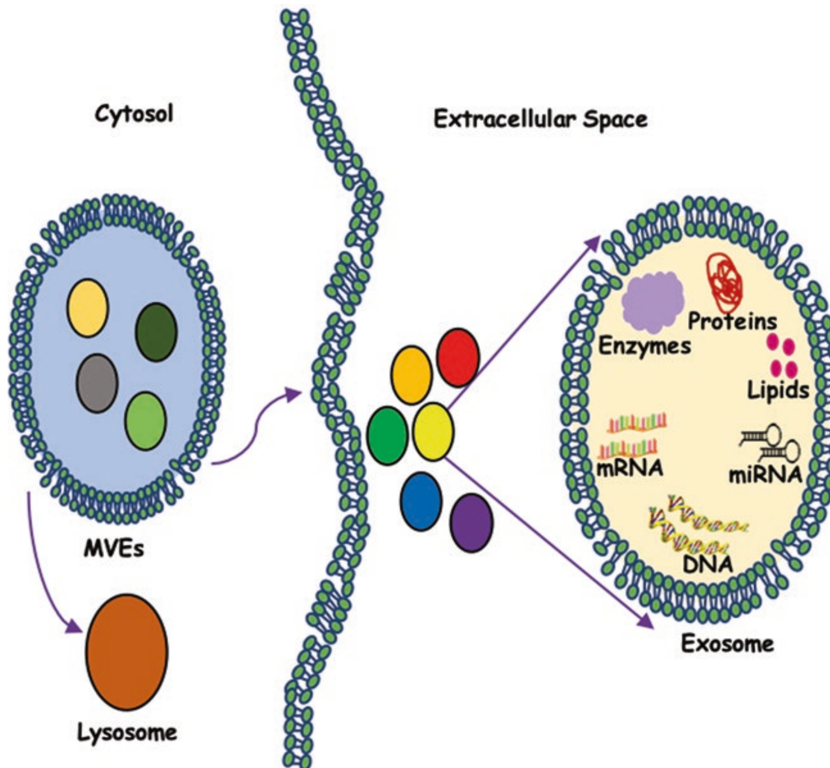

Fig. 5.4 A schematic model showing the release of exosomes into extracellular space

if the tumor is too large to fully resect, or if the tumor is causing damage to other organs [3]. These patients are given cycles of chemotherapy to initially shrink the size of the tumor. The amount of cycles depends on the size and severity of the tumor [3]. The typical combination of chemotherapy in this group is similar to low-risk patients. Carboplatin, cyclophosphamide, doxorubicin, and etoposide are generally used [30]. Once the tumor size has been decreased, surgery is the next step. In some cases, surgery is done prior to chemotherapy, to target the remaining tumor not resected in surgery [30]. Radiation is rarely used in the intermediate-risk group [3].

High-risk neuroblastoma patients have characteristics of metastasis or aggressive features of cancer cells [12, 17]. There are many ways to treat this category of neuroblastoma, and the most effective way has yet to be determined [12, 17]. The most common protocols include multiple phases of treatment: induction, surgery, consolidation, and maintenance [12, 17]. The induction phase focuses on reducing the size of the tumor and removing as much of the tumor as it is able in a quick manner [12, 17]. High dosages of chemotherapy are used, and the most common medications include cisplatin, etoposide, vincristine, cyclophosphamide, doxorubicin, and topotecan in a variety of alternating combinations [35]. Surgery is performed next to remove large amounts of the tumor [35]. After the surgery is performed, the next round of treatment begins to eliminate the body of any remaining cancer cells. Consolidation involves both chemotherapy and stem cell transplants. Research has shown that patients who are given high-dose chemotherapy followed by a stem cell transplant, have better results than straight chemotherapy [34, 35]. Stem cell transplant is autologous, so stem cells are harvested during the induction phase of therapy [34]. Also, stem cell transplants given back to back have shown promising results [34]. Once chemotherapy and the stem cell transplant are complete, radiation is done to the pri-

mary tumor site as well as any places of previous metastasis [35]. This ensures any left behind remnants are eliminated. The final phase in the treatment is maintenance, which is performed to prevent patients from relapse [35]. Treatments in this phase include medications to stimulate the immune system or to mature tumor cells [35]. Retinoid therapy using 13-cis-retinoic acid (isotretinoin) is most common, along with other immunotherapies [35]. Retinoid therapy causes cancer cells to differentiate into mature cells. Maintenance includes heavy monitoring of tumor relapse. Relapse occurs in approximately 50% of high-risk patients and 5–15% in low- and intermediate-risk patients. Normally, relapse occurs within the first few years after the initial treatment [35].

5.5 Neuroblastoma and Immunotherapy

Several studies have shown that the neuroblastoma microenvironment is immunosuppressive and tumor growth promoting. In recent times, many strategies are devised and tested to overcome this, and they are being developed to promote anti-tumor immunotherapy. The understanding of the biology of immunotherapy of neuroblastoma has increased a lot over the past 40 years [7, 8, 33]. Monoclonal antibody (MAb)-based immunotherapy, along with the discoveries in immune biology, has revolutionized the immunotherapeutic field to design more effective therapies for the treatment of high-risk neuroblastoma. These will be combined with new cytotoxic drugs and radiation therapies to improve survival and quality of life for patients with high-risk neuroblastoma [7, 8, 33].

Current therapy options for neuroblastoma are separated into three sections: induction, consolidation, and post-consolidation or maintenance therapy [33]. Treatment includes chemotherapy, surgical resection, and high-dose chemotherapy. Also included are stem cell rescue, radiation therapy, immunotherapy, and isotretinoin. The current treatment lasts approximately 18 months. The induction phase includes chemotherapy, stem cell collection, and surgery. The consolidation phase includes high dosages of chemotherapy and radiation therapy. The maintenance phase includes immunotherapy and retinoid therapy.

In immunotherapy, the patient's immune cells are used to recognize and destroy cancer cells. Monoclonal antibodies are used to recognize and attack a very particular neuroblastoma target cell [30]. Anti-GD2 (disialoganglioside) mAbs are a part of standard immunotherapy for high-risk neuroblastoma [38]. Dinutuximab (Unituxin) is a humanized monoclonal antibody that recognizes and binds to GD2 on neuroblastoma membranes; these antibodies in turn bind to Fc-receptors on the surface of granulocytes and NK cells and eliminate neuroblastoma cells through antibody-dependent cell cytotoxicity (ADCC) and cell-mediated toxicity. Dinutuximab is administered along with cytokine, interleukin-2 (IL-2). These will help the child's immune system seek out and demolish neuroblastoma cells. This is a new and advanced form of immunotherapy for children diagnosed with high-risk neuroblastoma. This antibody is usually given after all treatment options are exhausted, and a stem cell transplant has been done.

When diagnosed with high-risk neuroblastoma, it requires intensive treatment to achieve the current survival rate of slightly less than 50% [7, 8, 33]. With further research and a more robust understanding of the biology of neuroblastoma, there will be a way to identify factors that change the outcomes of patients who are diagnosed with this disease. Current research is focusing on further intensification of therapy to improve outcomes and evaluating the role of precision medicine in this patient population. With groundbreaking clinical trials and intense research into neuroblastoma, all possible options for treating patients who are diagnosed with this cancer are being explored, and the immunotherapy options are allowing for better hope for children diagnosed with this cancer.

5.6 Exosome-Mediated Therapeutics

Mesenchymal stem and stromal cells (MSCs) that have originated from multiple organs such as the bone marrow, umbilical cord (in-utero), adipose tissues, or placentas have shown to carry a therapeutic capacity of exosomes [36]. These were tested in numerous models of diseases. Exosome treatment has been compared to MSC treatment and has shown similar and even substantially better results [2]. When tested, MSC exosomes have exhibited favorable results that promote functional recovery and neurovascular plasticity. Some examples of these are subject, but not limited, to traumatic brain injury [40], the reduction in myocardial infarction size [1, 19], amelioration hypoxia-induced pulmonary hypertension [20], helping with the reparation of an injury to a kidney [13, 39], and the arrangement of neurological protection with the transfer of miRNA [18, 37].

Therapies that are exosome based portray a strong promise for the upcoming future in providing care for patients with various types of diseases. Patients with inflammatory diseases will especially benefit from exosome-based therapies [36]. The test for effectiveness in MSC-exosome treatments has been examined in multiple preclinical models. Safety concerns for the effectiveness of MSC-exosome treatment are of primary focus. All in all, though, cell-free exosome-based clinical trials are shown to have a slighter side-effect efficacy compared to live cell MSC trials [36].

5.7 Translational Advances

Exosomes could potentially play a role in the treatment of cancers. Exosomal vesicles can be used in a variety of ways to target different aspects of cancer, including diagnosis and treatment. Specifically, exosomes can be used as biomarkers for cancer diagnosis. Cancer cells are known to secrete more exosomes than healthy cells, which leads researchers to use this as a marker. The process of obtaining exosome samples from patients is fairly non-invasive, so this would be a clinically applicable way to help diagnose cancer. Not only can the exosomes be used as biomarkers, but also the proteins within the exosomes can be used to indicate cancer. Exosomes carry various molecules throughout the body, including proteins, miRNA, mRNA, etc. The overexpression of these molecules could be used as a potential prognostic factor for cancer. As well as a marker for cancer, exosomes could be used for treatment. Exosomes primarily function in cell communication, showing that they can interact with cell membranes to deliver their signals. This leads researchers to determine if exosomes could deliver drug therapy. Exosomes could be used as a method of delivering chemotherapy to malignant cells in the body. Also, it is thought that exosomes could be used to stimulate cytotoxic T cells into a response against cancer cells. The target of exosomes to stop tumor growth is also a consideration. Many studies have shown that exosomes display oncogenic and tumor-promoting effects. Therefore, the targeting of exosomes could be a key element to inhibiting tumor growth. Similarly, inhibiting the ability of cells to receive signals from the exosomes would also inhibit tumor progression. Although there are many possibilities of using exosome in cancer diagnosis and treatment, many mechanisms of exosomes are still undetermined. Further research into this field needs to be conducted.

5.8 Conclusion

Neuroblastoma is the most frequent solid tumor that is diagnosed in children under the age of 5. It develops in the immature nerve cells of the sympathetic nervous system during embryonic development. These tumors are most often found on the adrenal glands. Lumps in the abdomen or neck, bruising around the eyes, pain, fatigue, and weight loss are all common signs and symptoms of neuroblastoma. The current treatments for neuroblastoma involve a mixture of surgery, chemotherapy, radiation, retinoid therapy, and immunotherapy. Exosomes play an important role in the progression of this cancer. Exosomes

are small vesicles that are secreted by cells. They regulate cell communication, and transfer molecules between many cells in the body. Exosomes have been shown to progress tumor development, cause resistance to chemotherapy, and serve as a biomarker for tumors. Recent advances made in understanding the function of exosomes hold a promise to develop anti-cancer therapies.

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Conflict of Interest The authors declare no conflict of interest.

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6 COX-2 Signaling in the Tumor Microenvironment

Yuan Zhang, Sean Tighe, and Ying-Ting Zhu

Abstract

Tumorigenesis is a multistep, complicated process, and many studies have been completed over the last few decades to elucidate this process. Increasingly, many studies have shifted focus toward the critical role of the tumor microenvironment (TME), which consists of cellular players, cell–cell communications, and extracellular matrix (ECM). In the TME, cyclooxygenase-2 (COX-2) has been found to be a key molecule mediating the microenvironment changes. COX-2 is an inducible form of the enzyme that converts arachidonic acid into the signal transduction molecules (thromboxanes and prostaglandins). COX-2 is frequently expressed in many types of cancers and has been closely linked to its occurrence, progression, and prognosis. For example, COX-2 has been shown to (1) regulate tumor cell growth, (2) promote tissue invasion and metastasis, (3) inhibit apoptosis, (4) suppress antitumor immunity, and (5) promote sustainable angiogenesis. In this chapter, we summarize recent advances of studies that have evaluated COX-2 signaling in TME.

Keywords

Cyclooxygenase-2 · Structure · Prostaglandin · Arachidonic acid · Tumor · Tumorigenesis · Microenvironment · Regulation · Cell growth · Invasion · Metastasis · Apoptosis · Immunity · Angiogenesis · NSAID

6.1 Introduction

Tumorigenesis is a multistep and complicated process, in which oncogenes and tumor-suppressor genes are going through successive mutations and eventually lead to enhanced proliferation and resistance to apoptosis. Currently, several major hallmarks of human tumor have been universally reported, including evading growth suppressors, gaining genome instability, promoting replicative immortality, resisting cell death, eliminating cell energy limitation, promoting metastasis, inducing angiogenesis, sustaining proliferative signals, evading immune destruction, and aggregating inflammation [1, 2].

During the past few decades, the understanding of tumorigenesis has greatly increased [3] and the focus of studies has shifted from the malignant cells themselves to the tumor microenvironment (TME) and the interactions between them. TME, which consists of extracellular

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matrix (ECM) and cellular players such as fibroblasts, endothelial cells, neuroendocrine cells, adipose cells, leukocytes and so on, and their interactions [4], helps tumors to acquire their invasive characters. In detail, the tumoral niche has increasingly been reported to dictate abnormal tissue functions and play an important role in the subsequent evolution of malignancies [5]. Scientists have also found that a healthy microenvironment could help maintain the healthy cellular status and protect against tumorigenesis and metastasis [3]. Many studies have shown tumors are not only a mass of proliferative malignant cells, but they also attract other stromal cells [6], vascular cells [7], and immune cells [8] by secreting cytokines, chemokines, and stimulatory growth factors. These factors released by tumor cells may recruit other cells to rebuild the new microenvironment. Such communication between tumor cells and their microenvironment may enhance metastatic capability and immortal proliferation, causing eventual death [1, 2].

One of the key factors in the TME that has been characterized is cyclooxygenase-2 (COX-2). COX proteins are membrane-bound proteins, located on the nuclear envelope, and luminal side of the endoplasmic reticulum is an important mediator of angiogenesis and inflammation. It has three isoforms: COX-1, COX-2, and COX-3 [9, 10]. COX-1, which is expressed in most tissues, is a housekeeping enzyme to maintain the basal level of prostaglandins (PGs) [11]. It also helps maintain the internal homeostasis by regulating the processes such as vascular smooth muscle functioning, cytoprotection of the gastric mucosa, platelet aggregation, and renal function [9]. COX-3 is reported as a variant of COX-1, and it is mainly present in the central nervous system [12, 13]. By contrast, COX-2 is an inducible form, usually undetected in normal tissues and cells [14] in which its basal expression only can be found in the central nervous system, kidney, stomach [15], and female reproductive organs [16]. By contrast, it is usually constantly expressed in many types of tumor tissues [14, 17], such as squamous cell carcinoma, adenocarcinoma, transitional cell carcinoma, cholangiocarcinoma, hepatocellular carcinoma, and endometrial carcinoma [18, 19].

As TME actively participates in the tumor metastasis and progression, and COX-2 is one of the critical inflammatory mediators deregulated in many tumors, therapeutic strategies targeting the COX-2 in TME may have great potential and be highly selective. Below, we will highlight the role of COX-2 signaling in the regulation of tumor progression in the TME and discuss its potential value in tumor therapy.

6.2 Structure of COX-2

Human COX-2 is a homodimer of 581 amino acids, which encoded by COX-2 gene locates on the chromosome 1q25.2-q25.3 [20]. The dimerization of two 70 kDa subunits is necessary for catalytic activity and its own structural integrity [21]. Each subunit of COX-2 contains three domains to form the structure: a membrane-binding domain (residues 73–116), an N-terminal epidermal growth factor domain (residues 34–72), and a C-terminal catalytic domain which comprises the bulk of the protein [22–28]. The membrane-binding domain consists of four amphipathic α helices, three of which lie in the same plane, whereas the last one extends into the catalytic domain [29]. These helices have aromatic and hydrophobic residues. Therefore, this structure could create a surface that interacts with the lipid bilayer [22].

The peroxidase active site lies at the top of an L-shaped channel on the opposite side of the membrane-binding domain. It contains the heme that positioned at the bottom of a shallow cleft. Other molecules could access the heme easily except the dome formed by hydrophobic amino acids covers part of the cleft. At the entrance of the channel is a lobby. It is a large space that narrows to a constriction. Inhibitors or substrates can only pass into the channel when the lobby is open. On top of the lobby, the channel is surrounded by hydrophobic residues [25, 26, 28]. The structure of the active site makes COX-2 only react with specific substrate but not a wide range of organic hydroperoxides [30]. Interestingly, although the preference of the peroxidase relies on hydrophobic dome, mutation of the dome residues affects little on substrate specificity or peroxidase activity [31].

6.3 The COX-2 Signaling

6.3.1 The COX-2/PGE Signaling

COX-2 is a rate-limiting [20] and short-living enzyme [16] that converts phospholipase A2 (PLA2)-mobilized arachidonic acid (AA) into the signal transduction molecules thromboxanes and prostaglandins (PGs) [32]. One principal product of COX-2 is prostaglandin E_2 (PGE_2), a mediator contributing to the modulation of several biological processes, including angiogenesis, immunity, pain, and tumorigenesis [33–35]. In the tumor formation process, COX-2 could be overexpressed in TME due to transcriptional or posttranscriptional malfunction [36, 37]. Thus, COX-2 is an important marker for tumor identification [14, 38]. Elevated expression of COX-2 and its major product PGE_2 has been reported to be inversely associated with patients' survival rate [39–41].

Recent advances in the role of COX-2 and PGEs in the pathogenesis of cancer have been described [9, 15, 42–44]. The main form of prostaglandin involved in many types of cancers is PGE_2. PGE_2 can act on the receptors, for example, EP1, EP2, EP3, and EP4 to induce PGE_2 signal cascade, leading to changes of intracellular calcium, cAMP, and some inflammatory factors. As a result, physiological or pathological processes follow [45, 46]. Recent investigations support that PGE_2 may enhance progression of colorectal cancer [47–49], and EP4 is a therapeutic target for cancer therapy [50, 51]. COX-2-derived PGE_2 can also contribute to tumor development through several mechanisms including inhibition of apoptosis. However, the mechanisms by which PGE_2 regulates apoptosis are still largely unknown. The EP2 and EP4 receptors mediate their activities through cAMP production. Suppression of apoptosis by cAMP has been seen in intestinal cells through the induction of the IAP family member inhibitor of apoptosis 2 (IAP-2) [52, 53]. Therefore, further research is warranted to investigate the antiapoptotic effects of PGE_2 mediated through cAMP, which results in the induction of the IAP family member c-IAP2.

6.3.2 Cytokines and Other Compounds Regulating COX-2 Signaling

6.3.2.1 IL-1β and TNF-α

Cytokines and other compounds such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) may promote expression of COX-2 mRNA and protein in human colorectal fibroblasts, profoundly in cancer-associated fibroblasts (CAFs) [54–56]. When stimulated with the pro-inflammatory cytokines IL-1β or TNF-α, orbital fibroblasts express high levels of COX-2 and PGE_2 [57]. Scientists have found that IL-1β or TNF-α promotes synthesis of PGE_2 by 25-fold in human colorectal fibroblasts (CCD-18Co) and five human colorectal fibroblast strains obtained at routine colonoscopies [58]. Greater levels of IL-1β-stimulated COX-2 expression and PGE_2 synthesis in the cancer-associated fibroblasts could only be accounted for partially by increased COX-2 promoter and transcriptional activity in the cancer-associated phenotype. We have noted that IL-1β and TNF-α induce mRNA overexpression of COX-2 and promote production of PGE_2 in human colorectal fibroblasts, especially in CRC-associated strains [54, 59] at a rate at which COX-2 mRNA decays can be dramatically retarded in vitro by PGE_2 [60].

6.3.2.2 NF-κB

The nuclear factor (NF)-κB could also regulate the activation of COX-2 signaling in cancer cells [61]. The subfamily of NF-κB proteins has five members, including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel [18, 62, 63]. Among the subfamily, p65 plays a role in the regulation of COX-2 in cancer cells [64, 65]. NF-κB/ COX-2 signaling could be induced by protein kinase C (PKC) [66], TRIP4 [65], ERK1/2 [67], IL-1β [61], caspase-3 [68], and conditions like endoplasmic reticulum (ER) stress [69]. Inhibition of this signaling is mediated by annexin A5 [66] and miR-16 [70].

6.3.2.3 PKC and MAPK

Cytokines and growth factors induce COX-2 expression via protein kinase C (PKC) signaling.

Molecules that interfere with microtubules such as taxanes could induce COX-2 by activating PKC and mitogen-activated protein kinases (MAPKs). There are three related MAPK proteins including ERK1/2, p38, and c-Jun N-terminal kinase, which are contributed to the induction of COX-2 [71]. These members could mediate PKC effects on COX-2 signaling in cancer cells [72]. Combination of PKC and COX-2 inhibitors can synergistically inhibit melanoma metastasis [73]. Among the MAPKs, p38 [74] and ERK1/2 [75] are downstream molecules of COX-2. In addition, COX-2/P38 signaling favors angiogenesis [74] and is involved in cancer cell resistance to apoptosis [76].

6.3.2.4 Other Signaling

There are also many other cytokines and compounds which can regulate COX-2 signaling. One example is COX-2/STAT3 signaling, which contributes to the proliferation [77] and epithelial–mesenchymal transition (EMT) [78] of cancer cells by promoting the immunosuppressive microenvironment [75]. Another example is SDF-1a which plays a role in cancer cell metastasis and invasion through the stimulation of COX-2 by interaction with its receptor CXCR4 [79, 80]. All the research above suggests that COX-2 signaling is highly involved in the pathogenesis of cancer.

6.4 COX-2 Signaling in Tumor Microenvironment (TME)

6.4.1 COX-2 Regulates the Tumor Cell Growth

The cell behavior is controlled by complex signaling pathways. It is thought that the malfunction of these signaling pathways causes tumor cells to grow uncontrollably. Two major signaling pathways, Ras-MAPK and the PI3K/AKT signaling, are frequently shown to be deregulated in many human cancers, which can stimulate cell growth and survival when activated [81, 82]. There is a strong evidence showing that COX-2, together with PGE_2, are mediators of cancer cell growth through the above signaling [83]. PGE_2 derived from COX-2 can enhance cell survival through the PI3K/AKT and Ras-MAPK/ERK signaling. Aberrant activation of the COX-2/PGE_2 signaling might increase mutations in the above two signaling pathways, which could promote tumor progression [84–86]. Furthermore, there are other ways mediating cancer cell growth by COX-2. For example, activation of stromal cancer-associated fibroblasts (CAFs) and neutrophils by COX-2 can release proliferative signals on cancer cells [87, 88], and induction of aromatase cytochrome P450 (CYP19) by COX-2 contributes to the conversion of estrogen to estrogen quinones [89], which is involved in tumor proliferation [90].

Under physiological conditions, normal tissue can control cell growth by the action of antiproliferative signals, which is a crucial mechanism for maintaining homeostasis [1]. The membrane-bound ligands and soluble growth inhibitors are two kinds of key compounds of above signals to repress cell growth. Scientists have demonstrated two antigrowth signals that can restrain proliferation and maintain tissue homeostasis [1]. However, deregulation of the COX-2/PGE_2 signaling may limit the function of these signals by additional mechanism. The first antigrowth signals can maintain cells in G0 state to block proliferation and keep cell quiescence. For example, transforming growth factor-beta (TGF-β) can block cell growth by activation of cyclin-dependent kinase inhibitors and suppression of c-Myc [91]. Usually, cancer cells are insensitive to the suppressive effect of TGF-β due to inactivated mutations of the receptors or downstream signaling effectors [91]. One study showed that mutations of TGF-β receptor type II occur in colorectal tumors at a high frequency [92]. However, these mutations do not exist in all types of cancer cells. It is also reported that overexpression of COX-2 can downregulate the expression of TGF-β receptor type II, which means COX-2 signaling can prevent the receipt of antigrowth signals [93]. The second antigrowth signals are to initiate a terminally differentiated state [1]. Aberrant activation of pathways such as β-catenin/WNT signaling in colorectal tumors contributes to the blockage of normal differentiation and maintenance of progenitor state of

cancer cells [94]. Recently, evidence demonstrates that the COX-2 signaling can activate the β-catenin/WNT signaling to keep cells in a progenitor state [95]. Furthermore, when there is lack of β-catenin/WNT mutations, inappropriate activation of the COX-2/PGE_2 signaling could discourage cell differentiation.

In addition to the above function, the activation of β-catenin/WNT signaling by PGE_2 might also serve to the acquisition of the immortal phenotype [95], which means it can help the cancer cells to get limitless replicative potential. For example, colorectal cancer is thought to start from such immortal cells initiated by mutations in the β-catenin/WNT signaling. Scientists demonstrated that in intestinal crypts, the stem cells and progenitor cells are maintained by activating WNT signaling [94]. Mutations of components of WNT signaling in colorectal tumors result in the formation of an active β-catenin/T-cell factor (TCF) complex that can mimic WNT signaling. It is reported that COX-2/PGE_2 signaling may play a role in keeping the crypt in the progenitor phenotype by activating β-catenin/TCF complex in colorectal cancer cells [95]. Perturbation of the WNT signaling by deleting TCF4 in mice also leads to loss of the stemness in the small intestine [96]. This suggests that the WNT signaling could maintain the crypt stem cell phenotype in both physiological and cancer status.

6.4.2 COX-2 Promotes Tissue Invasion and Metastasis

COX-2 has been shown to be one of the critical metastasis progression genes [97] participating in the metastasis into the brain [98], bone [99], lymph nodes [100], and liver [101]. Factors like IL-11 induced by COX-2 are related to the cancer metastasis [99]. In order to achieve the invasion and metastasis, cancer cells must show an invasive phenotype of more motile status. They lose and detach themselves from connected cells within the tumor, move into extracellular matrix, and finally invade into blood vessels and lymphatics [102]. After escaping from the primary tumor tissue, cancer cells must then colonize the surrounding tissue or distant sites with the help of blood or lymphatics. Recently, the significance of COX-2 as a necessary mediator for dissemination of cancer cells was reported in an in vivo model of breast cancer metastasis to the lungs [103]. Using both pharmacological and genetic methods, this study demonstrated that COX-2 is one of the key "metastasis" genes which helps to mediate tumor development, invasion, and metastasis to other tissues.

There are many other studies demonstrating that COX-2 signaling plays critical roles in the metastasis processes—more specifically, promoting a more metastatic phenotype in colorectal tumor cells through its product PGE_2. For example, EGFR transactivation mediated by intracellular Src can stimulate the motility and invasion controlled by PGE_2 [104]. PGE_2 could also promote cytoskeletal reorganization and eventually lead to invasion and migration of colorectal cancer cells via PI3K signaling [105]. Overexpression of COX-2 can modulate the adhesive properties of intestinal cells [93] and increase the activity of matrix metalloproteinase (MMP) to promote tumor invasion [106]. Inhibition of this marker can prevent the metastasis of colorectal tumors in vivo in both human [107] and mice [108]. In addition, c-Met, also known as the hepatocyte growth factor receptor, is transactivated by PGE_2 through an EGFR-dependent pathway in colorectal cancer [109]. C-Met signaling is associated with the loss of cell contact and invasive growth [110]. Scientists found that COX-2, c-Met, and β-catenin coexist at the invasive edge of colorectal tumor [109]. The transactivation of c-Met can induce nuclear accumulation of β-catenin and increase expression and invasion of urokinase-type plasminogen activator receptor through Matrigel [109]. COX-2 can also induce β1-integrin that is related to cancer cell invasion [111, 112].

Furthermore, COX-2 can induce epithelial–mesenchymal transition (EMT) through factors like transcription-3 (STAT3) and miR526b [78, 113]. In cancer cells, EMT is thought to be a promoter of invasiveness [18]. Inhibition of EMT mediated by COX-2 occurs after usage of cannabinoids in cancer [114]. Interestingly, in the TME, the tumor maintenance and progression are only regulated by COX-2 secreted by the tumor

cells but not by other normal cells such as stromal cells [115, 116]. Therefore, these findings suggest that COX-2 plays an important role in tumorigenesis.

6.4.3 COX-2 Inhibits Apoptosis

Apoptosis, the cell death programming process [117], plays an essential role in controlling cell number and maintaining tissue homeostasis in normal tissue [118, 119]. Malfunction of this mechanism results in excessive cell number and survival rate, which can lead to tumorigenesis and its malignant progression [120–122]. COX-2 is related to suppression of apoptosis in many cancer types. The ability of COX-2/PGE_2 signaling to control apoptosis in tumor cells may depend on factors such as the TME and vary between cell types. In this signaling, several mechanisms have been reported. COX-2 contributes to the cancer apoptosis resistance through delaying G1 phase to slow the cell cycle [123]. It also induces the expressions of BCL-2 [124, 125], MCL-1 [126], and Survivin [127] and represses caspase-3 signaling [128].

First, overexpression of COX-2 might regulate the intrinsic apoptosis signaling by inducing the expression of BCL-2 and increase resistance apoptosis induced by butyrate in rat intestinal epithelial cells [93]. Later studies demonstrated that COX-2/PGE_2 might suppress apoptosis by increasing the expression of BCL-2 through activation of Ras-MAPK/ ERK signaling [129]. Other studies also indicated that COX-2 signaling controls apoptosis by inducing the expressions of BCL-2 [124, 125]. Second, scientists found that COX-2 is a critical mediator in apoptosis resistance by increasing the expression of MCL-1 [126]. Knockdown of MCL-1 would sensitize the lung cancer cells to apoptosis substantially. Moreover, the expression of MCL-1 could be significantly decreased when COX-2 was suppressed [126]. Third, it was reported that overexpression of COX-2 contributes to the expression and stabilization of Survivin, which is an inhibitor of apoptosis in non-small-cell lung cancer [127]. Suppression of COX-2 activity could induce degradation of Survivin and lead to lower cellular response to apoptosis pathways [127]. Fourth, scientists have reported that overexpression of COX-2 limited the cleavage of HuR and caspase-3, which reduced cell apoptosis in the paclitaxel-resistant oral cancer cells [128]. They also showed that inhibition of COX-2 increased apoptosis in paclitaxel-resistant oral cancer cells by activating of caspase-3, both in vivo and in vitro [128]. Furthermore, studies also demonstrate that COX-2/PGE_2 signaling might regulate apoptotic by involving in many other pathways. For example, it is reported that PGE_2 activates prosurvival signaling, such as ERK signaling [130], PI3K/AKT signaling [105, 131], EGFR signaling [132, 133], and cAMP/PKA signaling [134].

Other conditions like hypoxia could also contribute to the induction of cell death. For example, in colorectal tumor cells, COX-2/PGE_2 signaling could promote cell survival in hypoxia condition by activation of Ras-MAPK signaling [86], suggesting that COX-2 plays an important role in promoting the survival rate of cancer cells under difficult microenvironmental conditions. In addition, wild-type p53 is a suppressor of COX-2 in mediating apoptosis [18, 36]. Mutations of p53 in cancer cells would create a positive-feedback loop between COX-2 and itself. It might be a chemotherapeutic target for cancers [36, 135].

6.4.4 COX-2 Suppresses Antitumor Immunity

COX-2 signaling plays an important role in immune resistance and cancer immunotherapy. It regulates the immune response through recruiting immune cells into the tumor milieu to induce an immunosuppressive state [136]. Cancer cells can release COX-2/PGE_2 to the milieu to suppress immunological responses by blocking the activity of cytotoxic T lymphocytes [137]. COX-2/PGE_2 has also been shown to be a major modulator of macrophage activation for a long time [138]. One of the major populations of tumor-infiltrating immune cells is tumor-associated macrophages (TAMs). Reprogramming the TAMs of M2 toward M1

phenotype or impeding the process toward the pro-tumor M2 subtype is an anticancer strategy [44]. COX-2/PGE_2 signaling could promote macrophage differentiating to M2 subtype [139, 140]. Immune suppression regulated by macrophages is related to increased T-cell infiltration regulated by CD4+/CD25+ and decreased CD8+ T-cell function [44].

Overexpression of COX-2 promotes tumorigenesis by inhibiting proliferation of B-type and T-type lymphocytes, especially natural killer T cells, and subsequently limits immunosuppression of the host [141]. COX-2 inhibits the exposure of antigen-specific T cells to their cellular targets and promotes the expression of indoleamine 2,3-dioxygenase and interleukin-4 (IL-4) by tumor cells [44]. Scientists have demonstrated that COX-2/PGE_2 is the factor resisted to the cytotoxicity induced by active form of antigen-specific T cells [142]. It has also been shown that T-cell receptors (TCR) such as TCR NKG2D (natural-killer group 2, member D), Vγ9Vδ2 (Vδ2 gene with the co-expression of the Vγ9 chain), and CD16 are all inhibited by COX-2/PGE_2 [143]. Moreover, COX-2/PGE_2 helps the immune suppression mediated by cancer. They play an important role in promoting CD4+ and CD8+ T-cell differentiation and directly inhibiting the proliferation and effector functions of regulatory T cells [144]. Furthermore, it is reported that Treg cells inhibited effector T cells by activating COX-2 signaling and participated in cancer immunosuppression [145, 146]. The expression of COX-2 is also significantly related to Treg localization and prevalence [147]. In addition, expression of the forkhead/winged helix transcription factor (FOXP3) gene could also drive the suppressive activity of regulatory T cells.

Natural killer (NK) cells are a subpopulation of lymphocytes that take part in innate immunity. All types of PGE_2 receptors are expressed by NK cells, and PGE_2 derived from tumor is a critical barrier to the NK cell-mediated killing. It has been reported that the natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, NKp46, major NK receptors (NKRs), NKG2D, and CD16, could all be inhibited by PGE_2 [143]. In addition, the function of NK cells such as secrete interferon-γ (INF-γ), exert cytotoxic effects, and migrate are all inhibited by PGE_2 [148]. EP2 and EP4 are the major receptors acted by PGE_2 while inhibiting NK cells. And frondoside A, an EP4 antagonist, inhibits breast tumor metastasis by acting on NK cells and decreases IFN-γ production by NK cells [44]. Furthermore, MDSC presents in many cancer types and blocks adaptive immunity by inhibiting NK cells and the activation of CD4+ and CD8+ T cells [148, 149]. COX2 produced by tumor cells would maintain high level of MDSC, and subsequently block the tumor immunity. It has been shown to allow the proliferation of tumor cells without control from the immune system of the host [44].

Dendritic cells (DCs) participate in both innate and adaptive immunity. COX-2 is a crucial immunomodulator of DC activities [150], which can reduce DC ability to present antigens, express MHC class II molecules, mature, and activate T cells [151]. COX-2/PGE_2 has been demonstrated to decrease the cytokine production of antigen-presenting DCs, away from a type 1 T cell (Th1) profile, and eventually result in a reduced antitumor activation of cytotoxic CD8+ T cells [152, 153]. Meanwhile, it is reported that EP2 and EP4 receptor subtypes of PGE_2 may be targets of modulating DC activity [90]. For example, PGE_2 could increase interleukin-10 (IL-10) production, which can lead to downregulation of DC functions. These abilities of COX-2/PGE_2 signaling to suppress antitumor immune responses may allow malignant cells to escape immunosurveillance and promote tumor development.

6.4.5 COX-2 Promotes Sustainable Angiogenesis

COX-2 induced in tumor is associated with angiogenesis [154]. Inhibition of COX-2 suppresses corneal neovascularization in experimental lung and colon tumor growth [155]. COX-2 expression localizes in tumor epithelium [106], stromal fibroblasts [115], endothelium [155], and infiltrating immune cells [156]. It also promotes the production of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor [157]. It was demonstrated that expression of

COX-2 was critical for the induction of VEGF and the subsequent tumor angiogenesis in an Apc/COX-2 double-knockout mice model [158]. It is also reported that in COX-2 knockout mice, fibroblasts showed decreased level of VEGF mRNA and protein, together with lower vascular density compared to wild-type mice [115]. Consistent with this, in vivo studies have showed that homozygous deletion of COX-2 led to slower growth of tumor xenografts and lower tumor vascular density [115]. One possible mechanism is that COX-2 might promote tumor angiogenesis through the production of PGE_2, which has been reported to involve in endothelial cell spreading and migration by activation of Cdc42 and Rac [159]. PGE_2 has also been demonstrated to induce VEGF expression in colon cancer cells by activating HIF-1, one of the key regulators of VEGF expression [160]. Furthermore, PGE_2 has been reported to regulate vascularization though chemokine receptor signaling. For example, in vivo model showed that PGE_2 can enhance basic fibroblast growth factor-induced chemokine receptor-4 that is crucial for vessel assembly [161]. Moreover, PGE_2 can stimulate the expression of CXCL-1 in vivo, a pro-angiogenic chemokine [162].

In addition, COX-2 modifies molecules involved in endothelial trafficking with vascular mural cells/pericytes, an interaction critical to vessel stability [163–165]. Pericytes are found in all vascularized tissues, attaching to the walls of blood vessels [166]. They surround vascular endothelial cells and communicate with them by physical contacts and paracrine signaling along the length of the blood vessels [167, 168]. Increased expression of key modulator of pericyte PDGF-β or enhanced pericytes recruitment is characteristic features of tumor vasculature [169–171]. Moreover, when transplanting cancer cells into Nestin-GFP/NG2-DsRed mice, type-2 pericytes were recruited during the angiogenesis of the development of tumor, while type-1 pericytes did not penetrate [172]. COX-2, which modifies the proliferation and function of pericytes, plays a crucial role in vascular response to chronic microenvironmental stress [173, 174]. A study in 2006 demonstrated the function of COX-2 in vascular assembly in an orthotopic xenograft model by using the specific COX-2 inhibitor SC-236. The results showed that tumor growth was suppressed by SC-236 significantly in human Wilms' tumor [164]. All the evidence above suggests that COX-2 could promote sustainable angiogenesis in tumor.

6.4.6 Regulation of COX-2 Expression by the TME

Upregulation of COX-2 has been described in many different types of tumors [175]. It is reported that the TME is a promoter of COX-2 overexpression [36]. This overexpression is led by uncontrolled function of transcriptional or posttranscriptional levels [37]; therefore, it could be an important marker to identify tumor cells from normal tissues [14, 38]. Although PTGS2 (the gene-encoding human COX-2) mutations have not been described clearly, there are several known mechanisms which can promote expression of COX-2 in tumor cells. In general, the mechanisms can be divided into two types: oncogene activation and growth factor signaling deregulation. For example, it is reported that the hypoxic microenvironment can induce COX-2 expression in colorectal tumor cells [86]. This upregulation is mediated by HIF-1, a regulator of transcription in hypoxia. The same regulation dependent on HIF-1 has also been reported in lung cancer cells [176]. Other examples include activation of the TGF-β receptors [177], gastrin receptors [178], c-Met [179], β-catenin/WNT signaling [180, 181], and the Ras-MAPK pathway [85, 182]. In addition, COX-2 is a constituent of exosomes derived from tumor [183]. Cancer promoters [184], oncogenic viruses [61], proinflammatory cytokines [185], radiation [186], and chemotherapy [187] are all inducers of COX-2 expression in cancer cells.

6.5 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

For decades, significant progress has been achieved in the discovery of effective drugs for colorectal cancer. One of those is nonsteroidal

anti-inflammatory drugs (NSAIDs) which inhibit COX-2 [188, 189]. Examples of NSAID include aspirin, ibuprofen, naproxen, nimesulide, and sulindac acid. Different NSAIDs may act via different signaling pathways to interact with COX-2. For example, ibuprofen, indomethacin, and naproxen can bind the activity site of COX-2 and inhibit its activity reversibly, while aspirin acetylates the activity site of COX-2, attenuating its activity irreversibly. Some NSAIDs, for example, aspirin, can facilitate the effect of COX-2 inhibitors for treatment of stage III colorectal cancer [190]. In fact, aspirin may reduce colon cancer mortality in women by as much as 50% [191–193]. Recently, a hybrid drug KSS19, a combination of NSAID rofecoxib and cis-stilbene, has been found to be a potent COX-2 inhibitor, which inhibits colon cancer cell growth effectively [194].

Although COX-2 inhibitors are promising candidates for treatment of cancer, some concerns for treatment of cancer by COX inhibitors have been raised. For example, an elevated risk of myocardial infarction may be linked to its usage [195]. In addition, the extended use of nonselective NSAIDs is also associated with certain pathological symptoms, for example, abdominal pain, dyspepsia, gastritis, gastrointestinal bleeding nausea, and perforation of gastroduodenal ulcers [196]. Therefore, no major clinical trials of those inhibitors were successfully completed due to concerns of their adverse effects. Nonetheless, NSAIDs are effective in certain degrees for prevention and treatment of cancer. For example, a randomized trial demonstrated that NSAIDs are preventive for colorectal cancer with polyps [197, 198]. According to the results of large-scale trials, including the Adenomatous Polyp Prevention on Vioxx trial [199], the Adenoma Prevention with Celecoxib trial [198], the Prevention of Colorectal Sporadic Adenomatous Polyps trial [200], and colon polyp prevention trial [201], COX-2 inhibitors are effective for prevention of recurrence from sporadic colon cancer. Regular consumption of NSAIDs is also helpful for lowering the risk of colorectal, breast, lung, and prostate cancer [202]. In all, COX inhibitors have shown promise, but there are still safety concerns.

To decrease the risk from COX inhibitors, many researchers have used low dose of COX inhibitors with other NSAIDs that target other critical pathways in carcinogenesis. For example, combination of celecoxib with erlotinib (an EGFR tyrosine kinase inhibitor) is more effective to control polyp formation using an ApcMin/+ mice model and to inhibit cancer growth in a xenograft model [203]. Celecoxib with erlotinib treatment is more effective for treatment of the advanced non-small-cell lung cancer [204]. A 5-lipoxygenase inhibitor has been shown to inhibit resistant tumor cells to SC-236 (COX inhibitor) and tumor growth in a breast cancer animal model [205]. Combined treatment of celecoxib with peroxisome proliferators-activated receptor-γ agonist has been shown better than either alone in a mouse breast cancer model [206]. Combination of aromatase inhibitors with celecoxib has been shown better for patients suffering from metastatic breast cancer than either alone [207]. Therefore, we may like to reconsider the prospect of COX inhibitors for treatment of cancer.

6.6 Conclusion and Perspective

As studies have shown over the last few decades, COX-2 is one of the key markers indicating worse cancer prognosis and stimulates cancer via various roles in the TME. To date, clinical and basic research has shown that reduction of PGE_2 synthesis by either specific COX-2 inhibitors or NSAIDs has the potential to decrease the risk of tumorigenesis of certain types [97, 208–214]. Therefore, therapeutic strategies targeting the COX-2 in the TME may have great potential to improve clinical outcomes. COX-2 signaling in the tumor environment is summarized as follows (Fig. 6.1):

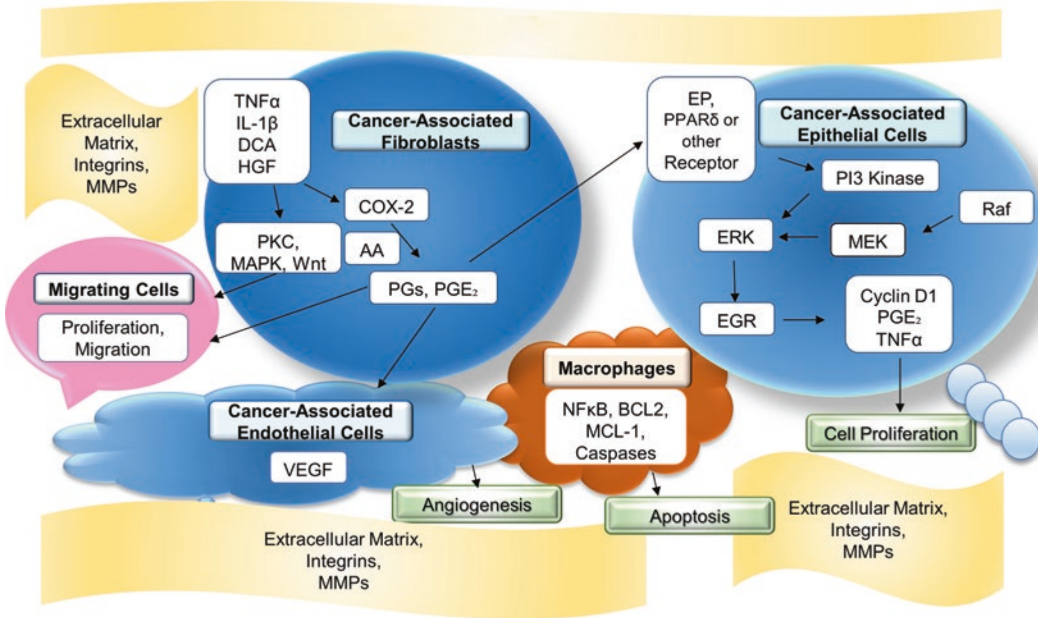

Fig. 6.1 COX-2 signaling in the tumor environment

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7 Renin–Angiotensin System in the Tumor Microenvironment

Kenta Nakamura, Ryuhei Okuyama, and Yutaka Kawakami

Abstract

For enhancing the antitumor effects of current immunotherapies including immune-checkpoint blockade, it is important to reverse cancer-induced immunosuppression. The renin–angiotensin system (RAS) controls systemic body fluid circulation; however, the presence of a local RAS in tumors has been reported. Furthermore, the local RAS in tumors influences various immune and interstitial cells and affects tumor immune response. RAS stimulation through the angiotensin II type 1 receptor has been reported to inhibit tumor immune response. Therefore, RAS inhibitors and combined treatment with immunotherapy are expected in the future. In this chapter, we provide a background on the RAS and describe the tumor environment with regard to the RAS and tumor immune response.

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Keywords

RAS · Cancer-associated fibroblasts · Immunotherapy · PD-1/PD-L1 · Tumor immune response · ARB · T cell · TGF-β · MDSC · Macrophage · Fibrosis · Angiogenesis · Hypoxia · ROS · COX-2

7.1 Introduction

Recent cancer immunotherapies, including immune-checkpoint blockade (i.e., the blockade of programmed cell death protein 1 (PD-1), programmed death-ligand (PD-L1), or cytotoxic T-lymphocyte antigen-4 (CTLA-4)), have produced durable clinical effects in some patients with advanced cancers. However, only a subset of patients responded to these therapies, and not all responses continued indefinitely. Unresponsiveness to immune-checkpoint blockade therapies may be mediated by numerous immunosuppressive mechanisms that inhibit antitumor T-cell responses and T-cell infiltration into tumor tissues [1, 2]. To improve current cancer immunotherapies, strategies to modulate various immunosuppressive cells, which are negative factors in immune-checkpoint blockade therapies, should be developed [2].

The renin–angiotensin system (RAS) is an endocrine system that is generally considered to

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systemically regulate hydromineral balance and blood pressure. However, recent data have demonstrated that renin and angiotensinogen genes and their products are locally expressed (the local RAS) at many tissue sites, where they serve as the fundamental regulators of many additional physiologic and pathophysiologic processes [3].

The main components of the RAS (i.e., angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), angiotensin I (Ang I), and angiotensin II (Ang II)) elicit their action through several receptors, including Ang II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). AT1R and AT2R are generally associated with inverse effects [3, 4]. Angiotensin II receptor blockers (ARBs) inhibit the effects of AT1R related to angiotensin II. Generally, AGT is produced and released into circulation by the liver and is then hydrolyzed to Ang I by renin in the juxtaglomerular cells of the kidney (Fig. 7.1). Subsequently, Ang I is hydrolyzed to Ang II by ACE in the endothelial cells of the lungs.

In the tumor microenvironment, the major components of the RAS are expressed in cancer cells as well as in stromal cells, such as macrophages and cancer-associated fibroblasts (CAFs) (Fig. 7.1) [3]. Additionally, the components of the RAS have been reported in endothelial cells, neutrophils, dendritic cells, and T cells [3, 5–8]. The local RAS in cancer tissues is involved in cellular migration, proliferation, inflammation, and angiogenesis in the tumor and the supporting stromal cells [9–11]. RAS antagonists have been found to suppress tumor progression in various experimental cancer models, and retrospective studies in humans have provided evidence that the long-term use of RAS inhibitors, such as ACE inhibitors and ARBs, may protect against cancer [3]. Additionally, the overexpression of the components of RAS is associated with tumor growth in breast cancer, ovarian cancer, and renal cancer [12–14]. Furthermore, signaling associated with AT1R may promote tumor growth [3, 4].

With regard to angiogenetic suppression, the combination of an anti-vascular endothelial growth factor (VEGF) antibody and RAS inhibitor was found to improve the survival rates of patients with metastatic renal cell carcinoma, metastatic colorectal cancer, progressive liver cancer, and glioblastoma [15–21]. Additionally, a meta-analysis reported that a RAS inhibitor might improve the survival rate of patients with cancer [22].

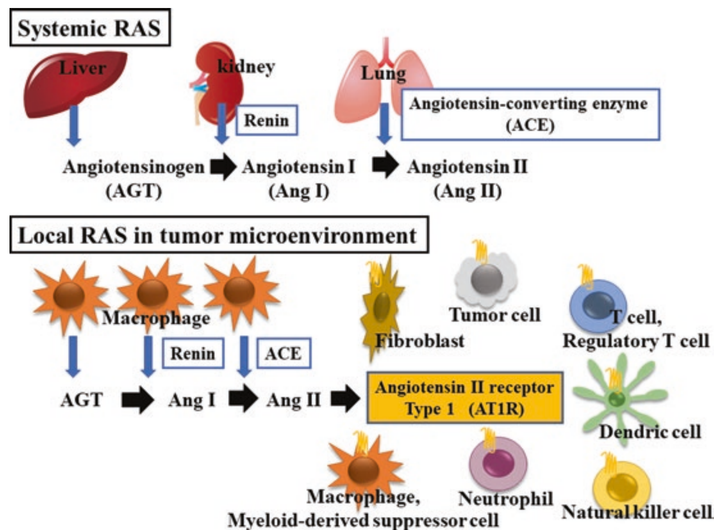

Fig. 7.1 The systemic and local renin–angiotensin system (RAS). Tumor-infiltrating immune cells, such as macrophages, neutrophils, T cells, dendritic cells, and natural killer cells, express the components of RAS, such as renin, angiotensin-converting enzyme (ACE), and angiotensinogen (AGT). Fibroblasts, which are interstitial cells, and certain tumor cells express angiotensin II type 1 receptor (AT1R)

7.2 Relation Between Immune Cells and the RAS

7.2.1 Cytotoxic T lymphocytes

Various in vitro studies have evaluated the role of the RAS in the induction of immune responses. RAS activation was shown to enhance antigen-specific T-cell responses, which can be blocked by ARBs [23]. Additionally, ARBs can inhibit the differentiation of human dendritic cells (DCs) from monocytes and their maturation by lipopolysaccharide stimulation [24]. In non-tumor-bearing mouse models, the RAS was found to be involved in the induction of T-cell responses [25]. However, few studies have reported on the role of the RAS in the induction of anti-cancer T-cell responses.

We previously reported that tumor antigen-specific T-cell responses increased with ARB administration [26]. ARB administration in C67BL/6 mice with the murine colon cancer cell line MC38 resulted in significant enhancement of tumor antigen gp70-specific T cells. Additionally, ARB administration did not change the number of $CD11b^{+}$ myeloid cells in tumors but significantly reduced their T-cell inhibitory ability and decreased the production of various immunosuppressive factors, including interleukin (IL)-6, IL-10, VEGF, and arginase, from $CD11b^{+}$ cells in tumors. Moreover, ARB administration decreased the expressions of immunosuppressive factors, such as chemokine ligand 12 (CXCL12) and nitric oxide synthase 2 (NOS2) in CAFs. Furthermore, the combination of an ARB and anti-PD-L1 antibody caused significant augmentation of antitumor effects in a $CD8^{+}$ T-cell-dependent manner.

Subsequently, a similar report was published. ARB administration (candesartan) increased the numbers of $CD3^{+}$ T cells and effector $CD8^{+}$ T cells and decreased the number of regulatory T (Treg) cells in 4T1 mouse mammary tumors [27]. Additionally, the effect of treatment with an anti-PD-1 antibody increased in a tumor model with reduced expression of the components of RAS. Furthermore, this was thought to be reinforced by a systemic tumor immune response because the effect of treatment with the anti-PD-1 antibody increased in a wild-type tumor transplanted on the other side of the murine model [27].

7.2.2 Regulatory T Cells

Treg cells are associated with the immune escape of cancer cells, and they inhibit tumor immune responses. The production of transforming growth factor-beta (TGF-β) in tumors and the low oxygenation of tumor tissues increase the number of Treg cells [28]. In a model of pancreatic cancer, ARB administration (losartan) was found to inhibit TGF-β, suppress fibrosis, and decrease the number of Treg cells [29]. In another model of pancreatic cancer, ARB administration was found to inhibit the activation of pancreatic stellate cells, decrease the expression of IL-1β, and decrease the number of Treg cells [30].

7.2.3 Macrophages

Macrophages can be divided into antitumor reactive M1 macrophages and immunosuppressive M2 macrophages. ARB administration has been shown to increase the number of M1 macrophages at the tumor site and increase the antitumor effect [31]. Generally, M2 macrophages are associated with wound healing, and they inhibit tumor immunity and aid in tumor growth [32, 33]. Various studies have reported on the change in M2 macrophages with RAS inhibition, and further analysis is necessary [34, 35]. Additionally, monocyte chemoattractant protein-1 (MCP-1) is produced for the stimulation of AT1R by tumors and interstitial cells [36]. MCP-1 that is produced correlates with the grade of invasion and tumor of the macrophages [36].

7.2.4 CAFs

The dominant mesenchymal cell components in tumor tissues are fibroblasts, which are strongly involved in cancer progression and metastasis

[37, 38]. CAFs are thought to induce an immunosuppressive tumor microenvironment through the production of various cytokines and chemokines that have impacts on tumor angiogenesis and remodeling of the extracellular matrix [39].

Ang II has been shown to stimulate the proliferation of CAFs and induce the production of various cytokines, such as TGF-β [40, 41], and some of these cytokines have immunosuppressive functions. CAFs, which are considered as immunosuppressive cells in the cancer microenvironment [37], have been shown to express AT1R [42].

CAFs are generally considered as immunosuppressive cells in the cancer microenvironment because they secrete and express immunosuppressive molecules, such as nitric oxide, TGF-β1, indoleamine-pyrrole 2,3-dioxygenase, PGE2, PD-L1, and PD-L2 [39, 43]. CAFs also produce CXCL12, which has been shown to inhibit T-cell infiltration into tumor tissues, leading to reduced antitumor effects of anti-CTLA-4 antibody and anti-PD-L1 antibody in a pancreatic cancer model [44].

We previously reported that ARB administration decreased the production of CXCL12 and NOS2 by CAFs [17, 26].

CAFs aid tumor growth through angiogenesis of the tumor microenvironment and immunocytic instructions via NF-κB signaling [45]. When the production of extracellular matrix by CAFs increases, tumor vessels are pressed and oxygenation reduces in the tumor [46].

ARB administration has been shown to decrease the production of immunosuppressive CXCL13 by CAFs [47]. CAFs have been found to cause dysfunction of T cells and natural killer (NK) cells [38], and TGF-β especially inhibits T-cell response and reduces tumor immunity [48].

7.2.5 Neutrophils

A high neutrophil-to-lymphocyte ratio has been shown to be associated with a poor treatment effect of immunotherapy [49, 50]. In a model of pancreatic cancer, ARB administration was found to inhibit the activation of pancreatic stellate cells, decrease the expression of IL-1β, and decrease the number of neutrophils [30].

7.2.6 Dendritic Cells

COX2 is induced by tumor cells and interstitial cells through stimulation of AT1R, and the antigen-presenting ability of DCs is inhibited through PGE2 [51, 52]. On the other hand, ARBs have been shown to inhibit the differentiation of human DCs from monocytes and their maturation by lipopolysaccharide stimulation [24].

7.2.7 Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) inhibit the activation of $CD8^+$ T cells [53]. Additionally, they induce increases in the number of Treg cells, the proportion of M2 macrophages, and the production of reactive oxygen species (ROS) [53]. ACE is associated with myelopoiesis and might be involved in the increase in the number of MDSCs; however, further study is necessary [54]. ARB administration (candesartan) has been shown to decrease monocytic MDSCs in 4T1 tumors and not alter granulocytic MDSCs [27].

7.2.8 NK Cells

COX2 is induced by tumor cells and interstitial cells through stimulation of AT1R, and the activity of NK cells is inhibited through PGE2 [52, 55].

7.3 Tumor Environment

7.3.1 Tumor Cells

In tumor tissues, Ang II, the main effector molecule of the RAS, acts through AT1R on both tumor cells and stromal cells and regulates the secretion of various growth factors and cytokines, such as IL-6, IL-8, and VEGF, partly through the

activation of NF-κB and signal transducers and activator of transcription family members [56, 57]. These molecules and transcriptional factors are well known to induce cancer-promoting inflammation and restrain antitumor immune responses (Fig. 7.2).

7.3.2 Fibrosis

Fibrosis of the tumor stroma inhibits immune cell invasion physically [58]. RAS inhibitor administration has been shown to inhibit the production of collagen I by CAFs (Fig. 7.2) [59]. Furthermore, when fibrosis of the tumor stroma is inhibited, the circulation volume in the tumor increases, low oxygenation improves, and tumor immune response increases [60].

7.3.3 Angiogenesis

RAS inhibitor administration has been shown to decrease the production of VEGF and reduce angiogenesis and vascular permeability [14, 61, 62]. Normalization of tumor vessels improves low oxygenation, reduces immunosuppression, and increases the effect of immunotherapy.

7.3.4 Hypoxia and ROS

AT1R stimulation reduces intratumoral circulation and causes hypoxia and acidosis (Fig. 7.2) [63]. Tumor hypoxia and acidosis result in the production of TGF-β [64, 65]. Tumor hypoxia causes dysfunction of T cells and DCs, increases the number of M2 macrophages and MDSCs, and increases the expression of PD-1/PD-L1 [32, 65–67]. Additionally, in the anoxic tumor environment, Ang II is produced.

AT1R stimulation induces ROS production from tumor and interstitial cells [68, 69]. ARB administration has been shown to decrease the production of intratumoral ROS [69]. Additionally, ROS has been found to induce Treg cells and tumor-associated macrophages and affect T-cell function [52, 70, 71].

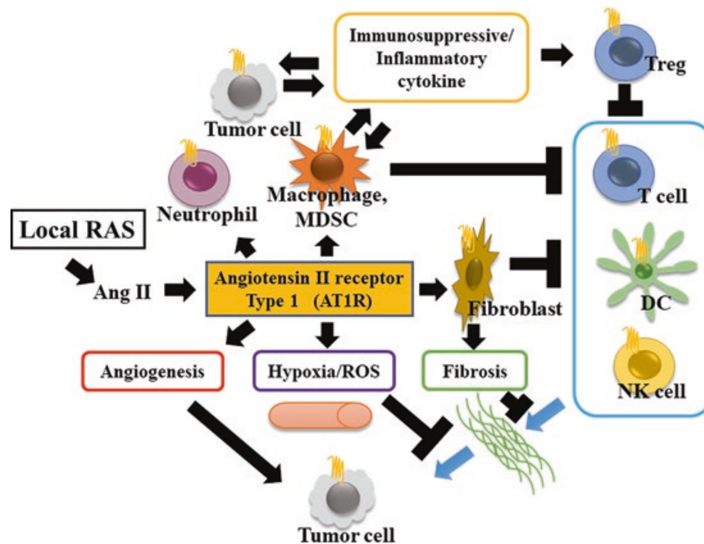

Fig. 7.2 The local renin–angiotensin system (RAS) inhibits tumor immune response through fibrosis, hypoxia, immunosuppressive cytokines, and immunosuppressive cells. Fibrosis of the tumor stroma associated with fibroblasts inhibits the movement of immune cells physically. Immunosuppressive cytokines and inflammatory cytokines associated with angiotensin II type 1 receptor (AT1R) stimulation promote further accumulation of immunosuppressive cells. Tumor growth is enhanced by angiogenesis of tumor vessels, but an immunosuppressive state occurs via anoxia

7.3.5 Immune Response Analysis in Clinical Trials

In a clinical trial for metastatic ductus pancreaticus cancer, RAS inhibitor administration was found to significantly increase overall survival [72]. Increased expression of genes associated with the processing and presentation of antigens was noted on genetic analysis of tumors from patients who received a RAS inhibitor [72].

7.4 Summary

As the local RAS in a tumor is complicated, contradictory results have been reported. However, RAS inhibition has been shown to improve tumor immune response. Thus, RAS inhibitors and combined treatment with immunotherapy are expected in the future. In tumors with low oxygenation, those associated with fibrosis resistance, and those with many immunosuppressive cells, RAS inhibitors might improve tumor immune response [73–77]. Additionally, RAS inhibitors might augment the treatment effect in renal cell carcinoma, colorectal cancer, and hepatocellular carcinoma, where inhibition of angiogenesis is beneficial. Moreover, a method to transport RAS inhibitors to the local tumor site has been studied, and it is expected that the tumor immune response will improve without an influence on systemic circulation [47]. It is hoped that further trials of the combination of RAS inhibitors and immunotherapy will be performed in the future.

Disclosure of Potential Conflicts of Interest The authors have no potential conflicts of interest to disclose.

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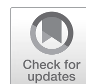

8 Stem Cell-Secreted Factors in the Tumor Microenvironment

Gema Jiménez, Julia López de Andrés, and Juan Antonio Marchal

Abstract

The importance of the microenvironment in tumor development and their resistance to drugs is increasingly well known. This microenvironment is composed of different cell types, among which cells with stemness properties such as cancer stem cells (CSCs) and mesenchymal stem cells (MSCs) are distinguished for their relevant role in tumor proliferation, angiogenesis, metastasis, and drug resistance. The relationship between these stem cells (SCs) and tumor microenvironment is conducted by the secretome, consisting of several factors, cytokines, chemokines, and hormones released to the surrounding stroma, which plays a deterministic role in tumor hallmarks. Knowing the intrinsic and complex communication network that SCs establish with the microenvironment will allow to address the tumor processes responsible for cancer progression and the generation of new targeted therapeutic approaches useful in the clinic arena.

Keywords

Cancer stem cells · Mesenchymal stem cells · Cancer-associated fibroblasts · Tumor microenvironment · Secretome · Growth factors · Cytokines · Extracellular matrix · Niche · Angiogenesis · Hypoxia · Metastasis · Epithelial-to-mesenchymal transition · Homing · Inflammation

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8.1 Introduction

Over the decades, tumor origin and development were attributed only to cancer cells; however, tumor cells do not act alone since they are immersed in a tumor microenvironment (TME) that comprises several cell types, a characteristic extracellular matrix (ECM) and a complex cytokines and growth factors network. The TME consists of the niche where the tumor develops, and it is unique for each tumor and patient, and also highly dynamic over time [1, 2]. The role played by TME in tumor hallmarks [3] such as high proliferation, invasion, angiogenesis, metastasis, and resistance to drugs [4–7] is increasingly well known. The TME contains several cell types including the tumor cells, cells from the immune system, CSCs, MSCs, fibroblasts, endothelial precursors, and a series of chemical components and biophysical signals [2]. This niche participates in the carcinogenesis by a complex network of cytokines, growth factors, and inflammatory and matrix-remodeling enzymes [8].

An essential process that must be given in the TME is the new vessel formation. Tumor neovascularization allows tumor growth and involves both tube-forming endothelial cells and their supporting pericytes, as well as tumor and stromal cells [9]. The new branched vessels of the existing vasculature and the development of neovascularization from endothelial cells and their associated pericytes or from cancer stem cells (CSCs) (in a process called vascular mimicry) depend on angiogenic signals from hypoxia regions or soluble factors from the TME [10, 11]. The resulting vasculature is chaotic and abnormally fulfills its functions, which facilitates the metastatic spread of cancer cells, increases hypoxia in the tumor [2, 8], and prevents the correct extravasation of immune cells and diffusion of drugs, helping tumor survival [12].

In TME development, cancer-associated fibroblasts (CAFs) are essential cells that secrete growth factors and cytokines, which stimulate the growth and survival of malignant cells [13–15] and contribute to drug resistance [16–18]. CAFs secrete also factors with chemoattractant properties, which stimulate the migration of other types of stromal cells and their progenitors to the TME, and promote angiogenesis by attracting pro-angiogenic myeloid cells and stimulating endothelial recruitment [19, 20].

Furthermore, the TME presents a wide diversity of infiltrating immune cells (IICs), among which are tumor-associated macrophages (TAMs), dendritic cells, lymphocytes, natural-killers, and neutrophils, which as a whole can perform both protumor and antitumor functions depending on a large extent on the signals from the TME [21]. IICs deliver to the TME growth mediators that stimulate the proliferation of both tumor and stromal cells and activate angiogenic processes [22]. Also, IICs promote invasive cellular phenotypes, contribute to therapeutic resistance, and improve protumor inflammation [5, 8, 23].

Beyond the contributions of different cell types to the TME, the ECM is another key component, and involves not only the physical scaffolding of the cells in the niche, but also a source of different factors and cytokines that model tumor behavior. CAFs, TAMs, and tumor cells secrete heparanases and matrix metalloproteinases (MMPs) that degrade the ECM, releasing these factors to the TME [14, 19, 24, 25]. Through them, ECM mediates in angiogenesis, inflammatory processes, dysregulation of stromal cells, and tumor proliferation [26].

In addition to the cell types described above in the TME, main role is played by characteristics SCs such as MSCs and CSCs. Both kinds of SCs have several common features and participate actively in the TME, being essential for tumor growth. In this chapter, we first present the similarities and specific characteristics of both SCs. Second, we describe the specific particularities of the secretome released by these cells and how it participates and regulates the TME and the pathogenic processes associated with tumor development.

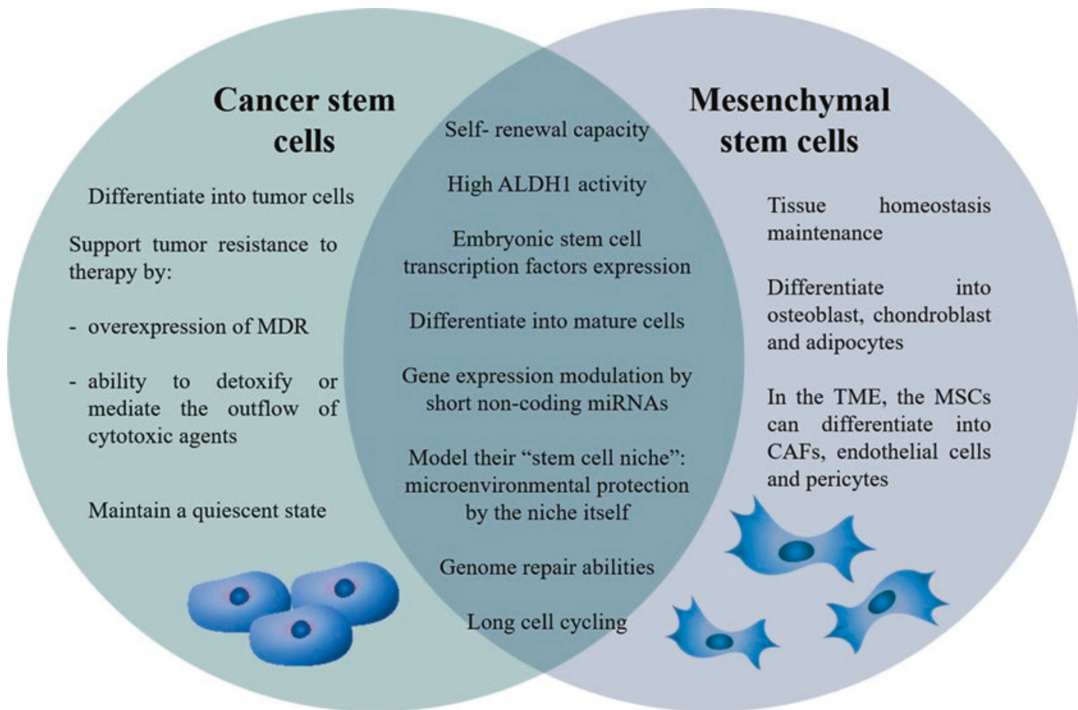

Fig. 8.1 Schematic illustration of the differential and shared characteristics of both stem CSCs and MSCs

8.2 Stem Cells in the Tumor Microenvironment

Stem cells are rare cells defined by the capacity to self-renew themselves and being able to differentiate into mature cells from a tissue [27]. In the TME, these cells will acquire special importance as responsible to manage its origin and particular characteristics (Fig. 8.1).

In the first place, although there are different proposals about how a tumor is generated, there is evidence of the existence of a minority subpopulation in the TME called CSCs, responsible for tumor growth, metastasis, and cancer recurrence [28]. CSCs present similar characteristics to MSCs in terms of their capacity for self-renewal, the expression of embryonic SCs transcription factors, similar regulation of several signaling pathways, and gene expression modulation by short noncoding miRNAs [29]. CSCs characterization is a complex challenge due to surface markers not being universal for any cancer type, the existence of heterogeneous CSC pools in the same tumor, and the instability of the phenotype [30]. However, several markers have been useful to identify CSCs like CD133 and CD44 [31], aldehyde dehydrogenase 1 (ALDH1) activity [32], and its ability to exclude Hoechst 33342 (side population) [33].

The importance of CSCs in the TME also lies in tumor recurrence and metastasis [34, 35]. Moreover, CSCs provide tumor resistance to radio- and chemotherapy due to the overexpression of membrane proteins of multidrug resistance (MDR) and their ability to detoxify or mediate the outflow of cytotoxic agents [33, 36], high ALDH1 activity [36, 37], rapid reparative response to DNA damage [38], and their ability to maintain a quiescent state [39]. However, CSCs require the TME to regulate their proliferation and self-maintenance, interacting closely with the cells that comprise it [40, 41]. It is known that CSCs not only get adapted to TME, but also contribute aggressively to its generation and cell composition; thanks to the development of a powerful interactive network composed of cytokines, growth factors, chemokines, hormones, miRNA, microvesicles, and exosomes through

which CSCs can recruit and activate different cells types like MSCs or vascular endothelial cells [41]. As well, the ECM is remodeled by the CSCs to maintain stem cell properties through anchorage, cell–cell and cell–ECM contact signals, and biomechanical properties [25].

On the other hand, one of the cell types recruited by the TME includes the MSCs, multipotent SCs that reside in many human organs and comprise a heterogeneous population with self-renewal ability [42]. Although their morphology, immunophenotype, and differentiation potentials are dependent on their tissue of origin [42], three criteria have been defined for their identification: (i) must be plastic-adherent when maintained in standard culture conditions, (ii) must express certain membrane markers, and (iii) must differentiate in vitro to osteoblasts, adipocytes, and chondroblasts [43, 44].

MSCs could be also found in the circulatory system and can arrive to inflammatory sites, where they seem to perform a restorative function, not only by structural repair of tissue, but also modulating the local environment due to its immunomodulatory and anti-inflammatory properties [42]. The role played by MSCs in the TME is not exempt from controversy [45]; however, in a relevant way, it has been shown that these cells are recruited by the TME [46–48]. It has been amply demonstrated that MSCs contribute to tumor growth and proliferation [48–51], increase the metastatic potential of tumor cells by promoting their motility, invasiveness [52, 53], the epithelial-to-mesenchymal transition (EMT) [54], and angiogenesis [55, 56], and participate in the appear of CAFs in the TME [51, 57]. Moreover, they play a key role in the tumor niche formation and support CSCs maintenance [58, 59]. Recently, our research group has shown that the MSCs secretomes, among which are interleukine-6 (IL-6) and hepatocellular growth factor (HGF) stand out, support the selection of CMCs with specific chromosomal alterations characterized by a translocation in the long arm of chromosome number 17 (17q25), that makes them more aggressive [58].

8.3 Stem Cell-Secreted Factors

In normal adult tissues, the presence of MSCs generates an environment termed as "stem cell niche," and the communication between the MSCs and their microenvironment is fundamental for normal tissue homeostasis, SCs maintenance, differentiation, and immunomodulation [60]. In cancer, this SC niche is modified with altered intercellular communication, be transformed in a TME that allows tumor growth changes over tumor progression and re-adapting [60–62]. All cells that constitute the TME display altered or modified secretomes compared to normal tissues, with simultaneous up- and downregulation of several factors [63]. SCs communicate with their microenvironment through the release of microvesicles and exosomes, as well as a wide range of soluble factors that include chemokines, cytokines, growth factors, hormones, and metabolites [64]. Specifically, factors released by tumor SCs promote several associated tumor processes, including tumor growth, invasion, metastasis, and promotion of angiogenesis, in addition to other processes such as influencing in cell phenotype, homing, differentiation, inflammation and immunodulation processes, and drug resistance mechanisms [63] (Fig. 8.2).

8.3.1 Angiogenesis

A decisive factor in tumor development is the presence of blood vessels, which provide both the nutrients and oxygen needed, and offer support for the metastasis. Several studies show that tumor SCs secrete vascular endothelial growth factor (VEGF), which is the principal growth factor promoting vascularization [65, 66]. Furthermore, it has been observed that the secreted VEGF itself has the potential to induce differentiation of MSCs into endothelial cells (ECs) [66, 67]. However, this factor not only has this fundamental role in the TME, but it also stimulates CSCs proliferation and maintenance through the stimulation of neuropilin-1, a core-

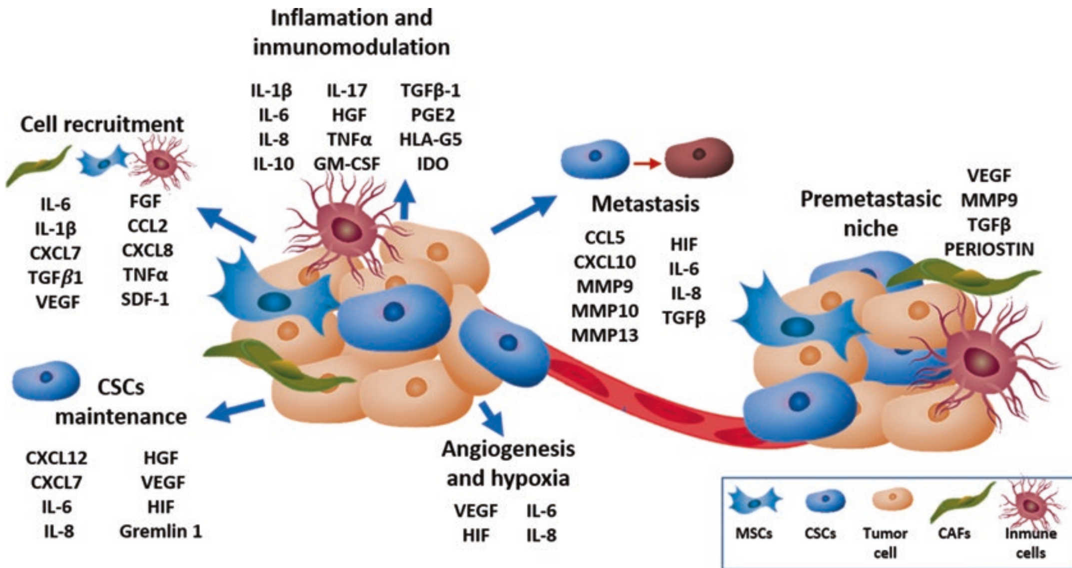

Fig. 8.2 Schematic overview of SCs secretome released to TME and the pathways and tumor processes it regulates

ceptor of VEGF receptor 2. In addition, VEGF overexpression accelerates tumor growth, promoting CSCs division [65, 68, 69] (Fig. 8.1).

Nevertheless, VEGF is not the only factor related with angiogenesis, IL-6 secreted by MSCs increases the secretion of endothelin-1 (ET-1) in cancer cells, which induces the activation of Akt and ERK pathways in ECs, leading to the development of mature vessels [70]. Also, a recent study situates another cytokine secreted by MSCs, the interleukin-8 (IL-8), as responsible for endothelial proliferation induction and tube formation, demonstrating the paracrine proangiogenic effect of IL-8 [71] (Fig. 8.1).

8.3.2 Hypoxia

A hallmark in solid TME is hypoxia, which is directly related with tumor progression and therapeutic response failure. Within the tumor, the oxygen concentration is variable, appearing in distinct areas with different oxygen contents. The responsible for the adaptation to hypoxic microenvironment is the hypoxia-inducible factor (HIF) family of transcription factors, and plays crucial roles in diverse tumor processes such as angiogenesis, treatment and immune system resistance, proliferation, tumor cell plasticity, metastasis, and maintenance of CSCs [72]. It was observed that under hypoxic conditions, MSCs increase HIF-1α secretion and their proliferative capacity. In addition, elevated release of energy metabolism-associated genes such as lactate dehydrogenase, GLUT-1, and PDK1 was observed, thereby leading to acidosis in the tumor microenvironment, and all this results in a feedback of the hypoxia environment [73]. On the other hand, the expression of HIF-1α and HIF-2α is different between non-SCs and CSCs. HIF-1α is produced by stem and nonstem tumor cells, and is only stabilized under acute hypoxic conditions, but HIF-2α is significantly secreted by CSCs and is accumulated under low levels of hypoxia or even normal physiological oxygen levels [74]; so, the role of the two HIF isoforms depends on the timely characteristics of the TME. In addition, HIF-1α produced by SCs stimulates tumor angiogenesis through the enhanced expression of angiogenic proteins like VEGF [75]. Also, several studies evidence that hypoxia plays a determinant role in CSCs maintenance, enhancing the self-renewal capacity, and retaining the undifferentiated state of CSCs, state that is reversible when normoxic conditions are resettled [76–78].

8.3.3 Metastasis

Cells from the primary tumor present intravasation capacity, which allows them to enter into the surrounding blood and lymphatic vessels, and around 0.2% of these cells survive in circulation and have extravasation ability; finally, they colonize distant organs producing metastasis [79]. As can be seen, metastasis is a very complex process that requires a set of factors that support it to achieve success. MSCs present different roles in the metastatic process, on the one hand, they increase the metastatic potential of tumor cells, and on the other hand, they present the ability to prepare the metastatic niche in the distant tissue [48]. Related to the increment of metastatic potential, the release of chemokine CCL5 by MSCs activates its receptor CCR5 on breast cancer cells thereby promoting altered breast cancer development and metastasis [52]. In addition, ovarian CSCs present CCR1, CCR3, and CCR5 upregulated, being more sensitive to CCL5 induction, enhancing invasiveness through nuclear factor κB (NF-κB) activation and the consequently elevated MMP9 secretion [80]. Others MMPs are highly secreted in the TME, such as MMP10 and MMP13 that are released by CSCs, and this fact promotes ECM degradation and remodeling, which enhances metastatic behavior [81, 82]. As other factors described, MMPs also perform different functions, such as MMP10 that has an essential role in CSCs maintenance and treatment resistance through the activation of Wnt signaling [83]. Main factors of other tumor processes also participate in metastasis, for example, hypoxia promotes metastasis through the activation and enhancement expression of HIF, which mediates paracrine signaling between cancer cells and MSCs mediated by CXCL10 and CCL5 and its respective receptors CXCR3 and CCR5 in cancer cells [84].

EMT phenomenon and the intravasation are essential processes in metastasis, and are processes driven by a complex network of cytokines and factors. For example, MSCs secretome in general, and IL-6, IL-8, and TGFβ in particular, have the capacity to upregulate EMT specific markers (N-cadherin, Vimentin, Twist, and Snail) via activation of PI3K/AKT pathway [85–87]. Once the cells are in the blood vessel, they have to perform the extravasation to be able to colonize the new tissue, and TGFβ displays an indispensable role in this process [88]. TGFβ induces angiopoietin-like 4 via the Smad signaling pathways in cancer cells, and these cells enter the circulation to metastasize to the lungs. After that, circulating cells that retain angiopoietin-like 4 release this cytokine and disrupt endothelial cell–cell adhesions in lung capillaries, facilitating the target organ invasion [89].

The TME also includes the metastatic niche, a niche in which there are also SCs and the factors secreted by them, making metastasis a successful process. Kaplan et al. first described the formation of a premetastatic niche where MSCs that express VEGFR1 present the capacity to migrate and form premetastatic niches through the production of MMP9, preparing it before the arrival and establishment of tumor cells [90]. Also, periostin (an ECM molecule) is highly expressed in CSCs [91] and when it binds to Wnt ligands, promotes stemness [92] so that the first CSCs that reach the premetastatic niche could favor the stemness of the new cells through this molecule. All these data together show that metastasis is a process induced by original TME secretome, where SCs are a principal player that can handle such complex processes as traveling through blood and lymphatic vessels and establishing a new tumor in a different organ.

8.3.4 Inflammation and Immunomodulation Processes

Inflammation and immunomodulation play a critical role in tumor development through the production of several molecules that participate in diverse tumor processes [93, 94]. In the TME there are several immune system cellular types including macrophages, neutrophils, mast cells, eosinophils, and myeloid-derived suppressor cell, which are attracted by TME through the tumor cell secretome, as well as ECM-degrading enzymes that allow invasion [75, 95]. The tran-

scription factors NF-κB and Stat3 regulate multiple aspects and serve as a central inflammatory mediator that responds to a large variety of immune stimulus [93, 94, 96], and as described in previous sections, these factors are very active in tumors. MSCs constitutively secrete several factors implicated in the immune suppressive role of these cells which include IL-1β, IL-6, IL-8, IL-10, HGF, TNFα, GM-CSF, TGFβ-1, prostaglandin E2, human leukocyte antigen-G5, and tryptophan-degrading enzyme indoleamine 2,3- dioxygenase (IDO) [97, 98]. In addition, CSCs have been demonstrated to have immunomodulatory properties through the release of inflammatory factors like IL-6, IL-17, and TGFβ, inducing Foxp3-positive regulatory T cells and pathogenic Th17 cells that can make the TME unresponsive to the recognizance of immune cells [99].

8.3.5 Homing

As described in the introduction section, the TME is composed of different cell types that interact to create the most optimal TME, as well as the different processes associated with tumor evolution; but to achieve this, it is necessary that the tumor "recruits" these cells. MSCs are attracted and activated by IL-6 released by different cell types, among them the CSCs, and in turn, the MSCs recruited produce CXCL7 that favors the maintenance of CSCs, generating a positive feedback loop [100]. Also, IL-1β, that shows higher expression in CSCs compared to their more differentiated counterparts [101], promotes MSCs migration through the expression of MMP1, which then activates the PAR1 and G-protein-coupled signal pathways [102].

Definitely, MSCs homing to tumor requires the participation of a complex molecule network that includes several cytokines and factors released by CSCs such as TGFβ1, VEGF, FGF, CCL2, CXCL8, and TNFα [103]. But MSCs do not respond only to signals from other cell types; for example, autocrine signaling of SDF-1 leads to the activation of Jak2/STAT3 and ERK1/2 signaling, thereby promoting FAK activation that finally promotes MSCs' migration to the TME [104]. TAMs also are critical modulators of the TME and support tumor progression; their recruitment is done through the chemokine CCL2 and its receptor CCR2, secreted by MSCs, as well as VEGF in a HIF-1α-dependent manner released by both SCs [105, 106].

8.3.6 Cell Phenotype Maintenance or Differentiation Induction

The maintenance or alteration of the cell phenotype or the stemness state is highly influenced by the TME. CSCs phenotype, proliferation, and invasiveness are regulated by MSCs and the CSCs themselves that activate NF-κB pathway through the release of several growth factor and cytokines, such as CXCL12, CXCL7, IL-6, IL-8, HGF, VEGF, HIF, and Gremlin 1 (see previous sections) [59, 100, 107, 108]. TGFβ is one of the key factors produced by the CSCs, and helps to transform fibroblasts and MSCs to cancer-associated fibroblasts (CAFs); thanks to the activation of TGFBR1/Smad pathways, and these CAFs participate in several TME process through its secretome network, like angiogenesis, EMT, and metastasis [62, 109, 110]. Moreover, MSCs also present the capacity to differentiate into pericytes and ECs under the effect of VEGF produced by both SCs [111]. The balance between the differentiated-dedifferentiated state of the CSCs is essential for tumor evolution and treatment resistance, and the balance between both states depends of NF-κB signaling (and related molecules describes above), enhancing Wnt activation that drives tumor cells dedifferentiation [112].

8.4 Future Trends

CSCs are responsible for tumor development, metastasis, and relapses, but the entire responsibility of a tumor process should not be associated only with a single cell type, since the TME is composed of different cell types that are interconnected by a complex network of chemokines, cytokines, growth factors, hormones, and metab-

olites. MSCs and CSCs create the stem niche and participate in several indispensable tumor processes such as angiogenesis, hypoxia, cell recruitment, inflammation, undifferentiated phenotype maintenance or cell differentiation, and metastasis. The potential of future therapeutic approaches is based on the knowledge of the TME, and especially of both types of SCs, as well as the complex communication network between them and with the rest of tumor subpopulations. For example, both SCs release VEGF to induce angiogenesis that supports CSCs maintenance and metastasis, and many novel approach drugs are focused on disrupting this growth factor pathway, including tyrosine kinase inhibitors [113, 114]. In the same way, high HIF expression correlates with poor glioma patient survival [115], so new therapies against this factor and its signaling pathway will allow the disruption of the hypoxic environment that affects several tumor processes and characteristics, including angiogenesis. A key factor in future therapeutic approaches is to avoid CSCs maintenance/protection. As described in this chapter, the entire TME in general, and the SCs in particular, has developed a complex cellular communication directed to CSCs preservation; therefore, new therapies should focus on this connection, and, in fact, there are already several studies and clinical trials aimed at these cytokines and specific factors, such as IL-6 [116, 117], IL-8 [118], HGF [119], and TGFβ [120]. In conclusion, the molecules released in the TME form a complex network that determines the success of the hallmarks of cancer [3], and may constitute a powerful tool in the therapeutic targeting of precision and personalized oncology.

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9 Tight Interplay Between Therapeutic Monoclonal Antibodies and the Tumour Microenvironment in Cancer Therapy

Quentin Riller, Aditi Varthaman, and Sophie Sibéril

Abstract

Therapeutic monoclonal antibodies (mAb) have changed the landscape of cancer therapy. With advances in the understanding of tumour biology and its microenvironment, different categories of mAbs have been developed; a first category is directed against tumour cells themselves, a second one comprises antibodies blocking the formation of neo-vasculature that accompanies tumour development, and, during the last decades, a third new category of immunomodulatory antibodies that target immune cells in the tumour microenvironment rather than cancer cells has emerged. In this chapter, we outline the main mechanisms of action of the different anti-tumour antibodies. We discuss the notion that, rather than passive immunotherapy that solely induces tumour cell killing, mAbs have multifaceted effects on the tumour microenvironment and could, qualitatively and quantitatively, reshape the immune infiltrate. We also discuss bystander effects of mAbs on the tumour microenvironment that should be carefully considered for the design of new therapeutic strategies.

Keywords

Immunotherapy · Monoclonal antibodies · Cancer therapy · Tumour microenvironment · IgG · Fab- and Fc-dependent mechanisms of action · Fc gamma receptors · Antibody-dependent cellular cytotoxicity · Antibody-dependent cellular phagocytosis · Innate immunity · Vaccinal effect · Long-term adaptive immunity · Immune checkpoints · Modulation of anti-tumour adaptive immunity · Bystander effects of monoclonal antibodies

9.1 Introduction

Forty years after their discovery by Milstein and Köhler, monoclonal antibodies are widely used for the treatment of cancer (Table 9.1). This success is partly due to the discovery of new therapeutic targets resulting from research advances in tumour biology and its microenvironment.

Lloyd Old and Ted Boyse's discovery of the first cell-surface differentiation antigens – used to

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Table 9.1 Approved or ongoing approval monoclonal antibodies in cancer regimen

International name	Class	Target	Indication	Approval year (EU/US)
Monoclonal antibodies targeting tumour-associated antigens				
Tositumomab-I131	Murine IgG2a	CD20	Non-Hodgkin lymphoma	NA/2003#
Rituximab	Chimeric IgG1	CD20	Non-Hodgkin lymphoma	1998/1997
Ibritumomab tiuxetan	Murine IgG1	CD20	Non-Hodgkin lymphoma	2004/2002
Ofatumumab	Human IgG1	CD20	Chronic lymphocytic leukaemia	2010/2009
Obinutuzumab	Humanized IgG1	CD20	Chronic lymphocytic leukaemia	2014/2013
Inotuzumab ozogamicin	Humanized IgG4 ADC	CD22	Acute lymphoblastic leukaemia	2017/2017
Moxetumomab pasudotox	Murine IgG1-immunotoxin	CD22	Hairy cell leukaemia	NA/2018
Blinatumomab	Murine bispecific antibody	CD19, CD3	Acute lymphoblastic leukaemia	2015/2014
Brentuximab vedotin	Chimeric IgG1 ADC	CD30	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012/2011
Gemtuzumab ozogamicin	Humanized IgG4 ADC	CD33	Acute myeloid leukaemia	2018/2017; 2000#
Daratumumab	Human IgG1	CD38	Multiple myeloma	2016/2015
Isatuximab	Humanized IgG1	CD38	Multiple myeloma	2020/2020
Alemtuzumab	Humanized IgG1	CD52	Chronic myeloid leukaemia	2001#/2001#
Trastuzumab	Humanized IgG1	HER2	Breast cancer	2000/1998
Pertuzumab	Humanized IgG1	HER2	Breast cancer	2013/2012
Ado-trastuzumab emtansine	Humanized IgG1 ADC	HER2	Breast cancer	2013/2012
Dinutuximab	Chimeric IgG1	GD2	Neuroblastoma	2015/2015
Edrecolomab	Murine IgG2a	EpCAM	Colon cancer	1995#/NA
Catumaxomab	Rat/mouse bispecific mAb	EPCAM/CD3	Malignant ascites	2009#/NA
Elotuzumab	Humanized IgG1	SLAMF7	Multiple myeloma	2016/2015
Mogamulizumab	Humanized IgG1	CCR4	Sézary syndrome	2018/2018
Polatuzumab vedotin	Humanized IgG1 ADC	CD79b	Diffuse large B-cell lymphoma	2020/2019
Sacituzumab govitecan	Humanized IgG1 ADC	TROP-2	Triple-negative breast cancer	NA/2020
Monoclonal antibodies that interfere with tumour–stroma interactions				
Cetuximab	Chimeric IgG1	EGFR	Colorectal cancer	2004/2004
Panitumumab	Human IgG2	EGFR	Colorectal cancer	2007/2006
Necitumumab	Human IgG1	EGFR	Non-small-cell lung cancer	2015/2015
Bevacizumab	Humanized IgG1	VEGF	Colorectal cancer	2005/2004
Ramucirumab	Human IgG1	VEGFR2	Gastric cancer	2014/2014
Olaratumab	Human IgG1	PDGFRα	Soft tissue sarcoma	2016/2016
Monoclonal antibodies that exert direct immunostimulatory effects				
Ipilimumab	Human IgG1	CTLA-4	Metastatic melanoma	2011/2011
Nivolumab	Human IgG4	PD1	Melanoma, non-small-cell lung cancer	2015/2014
Pembrolizumab	Humanized IgG4	PD1	Melanoma	2015/2014
Cemiplimab	Human IgG4	PD-1	Cutaneous squamous cell carcinoma	2019/2018
Atezolizumab	Humanized IgG1	PD-L1	Bladder cancer	2017/2016
Avelumab	Human IgG1	PD-L1	Merkel cell carcinoma	2017/2017
Durvalumab	Human IgG1	PD-L1	Bladder cancer	2018/2017

Source: '*The Antibody Society*' (https://www.antibodysociety.org/resources/approved-antibodies/)
Withdrawn or marketing discontinued, *NA* not approved, *ADC* antibody-drug conjugate

distinguish lineage and functional subsets of leucocytes [1, 2] – led to the CD (cluster of differentiation) classification and the wide use of cell-surface markers to distinguish between normal and malignant cells. Anti-tumour antibodies can be broadly classified into three categories: 1. Antibodies targeting CD antigens expressed specifically by tumour cells of the hematopoietic lineage: lymphocytes (CD20, CD22 CD38) and myeloid cells (CD30, CD33) (Table 9.1). 2. Antibodies that are directed against tumour-associated antigens (HER2/neu, MUC1, CEA, EGFR) – a number of molecules overexpressed by tumour cells discovered using tumour genetics [3–7] (Table 9.1). 3. Antibodies targeting the tumour microenvironment (TME). Established tumours are complex tissues composed not only of tumour cells but also of stromal and mesenchymal cells, vasculature components and immune cells. Tumour-derived factors stimulate blood vessel growth that in turn sustains tumour progression leading to the hypothesis that anti-angiogenesis agents might be an effective anti-cancer strategy [8–11]. The isolation of vascular endothelial growth factor (VEGF) – an endothelial-cell mitogen and a key regulator of angiogenesis in the TME – and the demonstration that an anti-VEGF mAb inhibits tumour growth in different preclinical models, led to the development and approval in 2004 of bevacizumab (humanized IgG1 anti-VEGF mAb) for clinical use in cancer patients [11, 12]. Other antibodies blocking neo-vasculature formation that accompanies tumour development have been subsequently developed, notably anti-VEGFR2, -PDGFRα mAbs (Table 9.1).

Control of tumour growth is largely dependent on the quantity and quality of the tumour immune infiltrate [13, 14]. The role of immunity in the control of tumours, although suggested as early as 1957 by Burnet [15] has been neglected for a long time. Studies in 1957 clearly showed that tumours harbour immunological determinants capable of eliciting anti-tumour immunity and long-term immune memory [16]. Consistent with these observations, the team of Boon reported that autologous cytotoxic T-lymphocytes (CTL) from melanoma patients recognize self-peptides derived from MAGE-1 protein expressed on tumours [17]. MAGE-1 is the first member of a larger family of proteins called the cancer testis (CT) antigen family – expressed only in tumours and in germ cells – which has been widely used in vaccination assays to elicit anti-tumour T-cell immunity. The use of genetically modified mouse models of immunodeficiency revealed the key role of immune components in tumour growth control; such as IFN-γ signalling, perforin molecules and the T-cell compartment. These preclinical data have incited interest in the understanding of cancer surveillance [18]. From the concept of ‘the three Es’ of cancer immunoediting defined by Schreiber’s group; elimination – corresponding to immunosurveillance of tumour growth by intratumoural immunity; equilibrium – representing the process by which immune attack induces the selection of resistant tumour cell variants; and escape – the process by which tumour cells escape immune control, came the finding that the immune system not only protects the host against tumour development but can also reshape the immunogenic phenotype of a developing tumour [18]. Studies performed on large cohorts of patients with cancer reveal correlations between the presence of tumour-infiltrating lymphocytes (TILs) and patient survival [13, 18, 19]. A favourable clinical outcome is often associated with the presence of tertiary lymphoid structures (TLS) – ectopic lymphoid formations that contain components required for the generation of an adaptive immune response including B-cell germinal centres, T-cell zones, mature dendritic cells and follicular dendritic cells [20]. These basic and clinical observations have paved the way to the development of a new category of immunomodulatory antibodies that target immune cells within the TME. Particularly, antibodies directed against regulatory receptors or immune checkpoint (ICP) molecules on immune cells have emerged over the last decade [21], as exemplified by the success of anti-CTLA-4 or anti-PD-1 antibodies in clinics. In the late 1990s, James P. Allison and T. Honjo (both awarded with the Nobel Prize in 2018) demonstrated, in preclinical tumour models, that the expression of inhibitory ICP on intratumoural T cells dampens

their anti-tumour activity and that blockade of PD-1/PD-L1 or CTLA-4 pathways using mAbs dramatically halts tumour development [22, 23]. These pioneering studies reveal anti-ICP mAbs as a promising strategy for specific tumour immunotherapy and have revolutionized the landscape of cancer therapy.

In this chapter, we will present the Fab- and Fc-dependent mechanisms of action of different categories of anti-tumour antibodies.

9.2 Direct Fab- and Fc-Dependent Mechanisms of Action of Therapeutic Monoclonal Antibodies in Cancer

Initially, it was thought that anti-tumour antibodies acted by rapidly recruiting blocking/killer mechanisms on tumour cells. Thus, use of mAbs was considered until recently more as passive immunotherapy based on their transient ability to block cancer cell activation and/or proliferation (i.e. by targeting growth receptors such as EGF-R or HER2/Neu (erbB-2)), to induce apoptosis (even marginal by HER2/neu, CD20), or to interfere with the adhesion of tumour cells (EpCAM) blocking the formation of metastases. However, the expression of mAb-targets on cancer cells and in the tumour area is not necessarily predictive of response to treatment. For example, although the presence of EGFR-positive tumour cells is a requirement for colorectal cancer patients to receive cetuximab and panitumumab (anti-EGFR mAbs), EGFR expression at the protein or mRNA level has not been correlated with treatment response [24], suggesting that the mechanism of activity of these mAbs may also be related to their effect on tumour infiltrating immune cells. Similarly, although the use of antibodies targeting the VEGF pathway has shown clinical benefits associated with a reduction in tumour blood vessel density, the direct neutralization of VEGF-driven vascular effects explains only part of their therapeutic effect. VEGF inhibitors, particularly bevacizumab, not only induce vessel normalization – associated with increased tumour blood perfusion, restoration of adhesion molecules on endothelial cells and improved influx of leucocytes into the tumour – but also activate and modulate the function of immune cells within the TME [12, 25]. Anti-VEGF and/or anti-VEGFR mAbs have an impact on the frequency of regulatory T cells and of tumour-infiltrating myeloid-derived suppressor cells (MDSCs) and reinvigorate dysfunctional DCs [12, 25].

Part of the therapeutic effects of anti-angiogenic antibodies can be triggered by Fc/FcγR interactions. Most of the marketed therapeutic antibodies are human IgG1 (either chimerized, humanized or fully human antibodies), the most efficient human IgG subclass, together with IgG3, in engaging FcγR and activating the complement cascade. Anti-tumour antibodies can trigger effector mechanisms leading to tumour cell death, such as complement-dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC), antibody-dependent cell phagocytosis (ADCP). The activation of the classical pathway of complement through the binding of C1q to the Fc portion of mAbs and the recruitment of Fcγ receptors (FcγRs) expressed by NK cells, neutrophils, monocytes and macrophages leads to the formation and/or the release of effector molecules (membrane attack complex made of C5b-C9, perforin and granzymes, TNF-α, reactive oxygen intermediates (ROI), etc.) that induce cell death. ADCC and ADCP in myeloid cells through the engagement of FcγR are considered to play an important role in the in vivo efficacy of anti-tumour antibodies both in pre-clinical tumour models and in treated cancer patients [26]. Macrophages in tumour tissues are important for the efficacy of therapeutic antibodies thanks to their expression of different types of FcγR, enabling ADCP. Several studies provide evidence that macrophages are effector targets of therapeutic antibodies in cancer; in vitro human macrophages phagocytose tumour cells in response to

anti-CD20 (rituximab) and anti-HER2/neu mAbs (trastuzumab) [27–29] and, in vivo, macrophages have been associated with a better response to trastuzumab [30, 31]. Interestingly, all human IgG subclasses, including isotypes that exhibit low NK cell-mediated ADCC due to their poor binding to FcγRIIIa, have the potential to engage other FcγR (FcγRI and FcRγIIa) expressed in macrophages and to stimulate macrophage-dependent phagocytosis [32]. Significant correlations of FcγR polymorphisms with clinical outcome in patients treated with rituximab [33, 34], trastuzumab [35, 36] and cetuximab [37, 38], argue in favour of a role for FcγR⁺ immune cells in the TME in the clinical response to mAb-based treatment. However, studies reveal no such associations in patients with breast cancer [39], raising the possibility of additional immune mechanisms that account for the clinical benefit of mAb-based immunotherapy. Notably, the duration and strength of the clinical responses following mAb treatment can be linked to the ability of tumour antigen-specific mAb to elicit adaptive cellular immunity via the activation of antigen presenting cells, as described later in this chapter.

FcγR/FcR interactions are also implicated in the anti-tumour activity of anti-ICP mAbs [40]. One underlying mechanism of the anti-tumour activity of anti-GITR, -OX40, -CTLA-4 and -TIGIT antibodies is through intratumoural depletion of regulatory T cells via FcγR⁺ myeloid effector cells [40–46]. Consistent with these preclinical studies, melanoma patients with higher frequencies of FcγRIIIA⁺ myeloid effector cells in the peripheral blood show higher response to ipilimumab treatment which is attributed to ADCC/ADCP mediated depletion of Treg in the TME [45]. Recently, Waight et al., reported an FcγR-dependent mechanism of action of anti-CTLA-4 mAbs that is independent of Treg depletion [46]. Engagement of activating FcγRIIIA on APCs by anti-CTLA-4, anti-TIGIT and anti-CD45RB antibodies mAbs improves T-cell activity by modulating both TCR and CD28 signalling [46].

9.3 Therapeutic Antibodies Reshape the Tumour Microenvironment

9.3.1 From Tumour Cell Destruction to the Triggering of Long-Term Adaptive Anti-tumour Immunity

In addition to the anti-tumour effects triggered by mAbs treatment on innate immunity, evidence suggests that these agents might also affect the local inflammatory and immune microenvironment [13]. Clinical data and in vivo animal models suggest that antibody treatment leading to tumour cell killing induces long-term anti-tumour responses by triggering target-specific adaptive memory responses, a phenomenon that has been termed the ‘vaccinal’ effect of antibody treatment [47]. Specific T- and B-cell responses are reported in cancer patients following therapy with anti-CA125 [48], anti-MUC1 [49], anti-HER2/neu [50, 51] and anti-EGF-R [52] mAbs. Studies in murine models also report that the therapeutic effect of anti-CD20 [53–56], anti-HER2/neu [57–60], or anti-EGF-R [61] mAbs depends on the induction of an adaptive immune response and on the presence of T cells. The anti-HER2/neu studies reveal an antibody-mediated mechanism in which danger signals activate both innate and T-cell-mediated immune responses [57–60]. A role for dendritic cells (DC) and macrophages at the tumour site in this vaccinal effect is supported by the ability of these cells to internalize – in an FcγR-dependent manner – exogenous IgG-complexed antigens (probably derived from tumour cell debris), and to present MHC II and MHC I-restricted peptides derived from these complexes [62–65]. In a human glioma model, FcγR-dependent engulfment of cetuximab-coated glial tumour cells by DCs leads to an increase in anti-tumour CD8⁺ T cells [65]. Several studies demonstrate that upon mAb therapy, a cross-talk between NK cells and DCs can occur [52, 66, 67]. Cetuximab-activated NK cells result in enhanced cross-presentation of EGF-R-derived peptides to specific CTL [52].

Interestingly, it has been reported that human macrophages and DCs equally present tumour-associated antigens to CD8^{+} T cells after phagocytosis of γ-irradiated melanoma cells [68]. One can thus hypothesize that phagocytosis of mAb-coated immune complexes by FcγR^{+} macrophages also leads to an efficient activation of CD8^{+} T cells. Nevertheless, the extent to which both APCs process and cross-present non-mutated tumour-associated antigens within the tumour microenvironment to prime T cells in situ has yet to be clarified. Non-mutated self-proteins overexpressed by tumour cells are universal target antigens to induce tumour-specific T-lymphocytes without the need to identify the mutanome of tumour cells. Recent results demonstrate that thymic deletion prunes but does not eliminate self-specific CD4^{+} and CD8^{+} T cells, and that some self-peptide-specific T cells can be detected at frequencies similar to T cells specific for non-self-antigens [69–72]. We also found that CD4^{+} T cells against non-mutated human CD20-derived peptides are present in healthy donors and lymphoma patients [73]. While T-cell responses against these self-derived epitopes can be limited by a self-tolerant T-cell repertoire, it has been demonstrated that anti-CA125, anti-HER2/neu, anti-MUC1 and anti-EGFR mAb treatment can circumvent this tolerance as shown by the increase in frequencies of CD4^{+} and/or CD8^{+} T cells recognizing peptides derived from the target molecule in cancer patients [48–52].

9.3.2 Effects of Immunomodulatory mAbs on Lymphoid and Myeloid Compartments Within the Tumour Microenvironment

In the last decade, therapeutic mAbs directed against inhibitory checkpoints have changed the landscape of cancer therapy. Clinical studies have demonstrated that these antibodies can induce durable clinical responses even in patients with advanced cancer [74–76]. Of the many different checkpoint receptors, the cytotoxic T-lymphocyte antigen-4 (CTLA-4), as well as PD-1 and its ligands, PD-L1 and PD-L2, are most intensely studied. CTLA-4, expressed on T cells, is an early contributor to the development of immune tolerance. It negatively controls the priming and early antigen-dependent T-cell activation in lymphoid organs, and is also expressed in regulatory T cells (Treg). CTLA-4 inhibition is used with the aim of stimulating T-cell activation and, subsequently, anti-tumour immune responses. Ipilimumab, a human IgG1 anti-CTLA-4 mAb, which was the first anti-ICP mAb to demonstrate survival benefit for patients with metastatic melanoma, received Federal Drug Administration (FDA) approval for melanoma treatment in 2011 and is currently in clinical trials in various cancers, including lung, colorectal, bladder, renal and prostate cancer (https://www.cancer.gov/about-cancer/treatment/clinical-trials/intervention/ipilimumab?pn=4). PD-1 is a checkpoint inhibitor of T cells within peripheral tissues and the tumour microenvironment. PD-1 is also highly expressed in intratumoural Treg cells and might enhance the immunosuppressive activity of these cells. MAbs that target the PD-1/PD-L1 axis are approved for the treatment of patients with melanoma, cutaneous squamous cell carcinoma, non-small-cell lung cancer, bladder cancer and Merkel cell carcinoma (Table 9.1).

Overall changes in the tumour microenvironment during ICP therapy, both in preclinical models and in treated patients, have been comprehensively analysed through longitudinal gene expression studies as well as high-dimensional profiling approaches, such as mass cytometry and single-cell RNA sequencing [77–82]. Major changes in tumour- and immune-associated genes are reported in melanoma patients who exhibit clinical activity following ipilimumab (anti-CTLA-4 mAb) therapy [79]. A lower expression was observed for genes encoding tumour antigens (e.g. members of the MAGEA family, NY-ESO-1, MLANA), for genes involved in dermatological phenotype and functions (e.g. SOX10, MITF, two key transcription regulators in melanocytes, and tyrosinases TYR and TYRP1, involved in melanin synthesis) and for genes implicated in cell growth and differentiation (e.g. MYC, MXI1, IGF1R, CDK2, CCND1,

BIRC7, HRK and TNFRSF10B). By contrast, many IFN-γ-inducible genes and Th1-associated markers (e.g. PRF1, TAP1 and GZMB) increased after ipilimumab treatment, suggesting an accumulation of this type of T cells at the tumour site, which might play an important role in mediating the antitumour activity of ipilimumab [79].

It is generally assumed that ICP blockade (anti-PD-1, anti-CTLA-4 mAbs) can restore antitumour activity in dysfunctional infiltrating immune cells. Checkpoint inhibitors amplify preexisting T-cell responses, broaden the range of antigens being targeted by the T-cell repertoire, and induce T-cell-mediated immune responses against tumour neoantigens [82–85]. A whole-exome and transcriptome analysis in tumours from patients with advanced melanoma treated with nivolumab (anti-PD-1 mAb) shows that mutation and neoantigen load reduce from baseline in responding patients [77]. Interestingly, in responding patients, T-cell clones expand in proportion to the number of neoantigen mutations that disappear on therapy, suggesting an effective immune elimination of tumour cells containing non-synonymous mutations and neoantigens, and a selective pressure against the generation of antigenic mutations.

Analysis of changes in the TME in tumours of mice treated with anti-CTLA-4 and/or anti-PD-1 mAbs by mass cytometry and single-cell RNA sequencing demonstrates that anti-ICP mAbs induce both quantitative and qualitative changes in intratumoural $CD4^+$ and $CD8^+$ T cells as well as NK cell subsets. The dramatic reduction in Treg frequency and suppressive functions, and the remodelling of the $CD4^+$ and $CD8^+$ T-cell compartments, lead to increased expression of an anti-tumour effector gene signature (e.g. *Ifng, Gzmb*). This study also shows that anti-ICP therapy induces a shift towards a more activated $CD4^+$ T-cell compartment that expresses high levels of IFN-γ. T-cell activation markers are also altered: anti-CTLA-4 decreases the expression of TIM-3, LAG-3 and PD-1 in tumour neoantigen-specific $CD8^+$ T cells, while anti-PD-1 therapy decreases the expression of LAG-3 and PD-1 [80]. Recent work from the Allison group in murine tumour models and human melanomas show that the clinical activity of anti-CTLA-4 or anti-PD-1 mAbs relies on distinct effects on intratumoural T-cell subsets [81]. Both antibodies induce the expansion of specific tumour infiltrating T-cell subsets. Anti-PD-1 mAb predominantly expands exhausted tumour infiltrating $CD8^+$ T cells, while anti-CTLA-4, but not anti-PD-1, modulates the $CD4^+$ T-cell compartment, particularly by expanding an $ICOS^+$ Th1-like $CD4^+$ effector subset. Differences in the impact of the two mAbs on specific subsets of lymphoid cells are also reported in the work of Gubin et al. [80].

Recent studies suggest that durable clinical responses to immunotherapy also depend on bystander effects on T-cell subsets that do not express ICP molecules. Indeed, $PD\text{-}1^+$ $CD8^+$ T cells have limited potential to give rise to a long-lasting effector response due to their acquisition of a stable epigenetic state that cannot be reverted by ICP blockade [86–90]. In a preclinical model of colon cancer, Kurtulus et al. examined changes in the RNA profiles of intratumoural $CD8^+$ T cells after TIM-3/PD-1 blockade [91]. Two TIL populations with either high ($PD\text{-}1^+TIM3^+$) or low ($PD\text{-}1^-TIM3^-$) dysfunctional state acquired an effector profile following TIM3/PD-1 blockade. Interestingly, the $PD\text{-}1^-TIM3^-$ subset showed more profound changes than $PD\text{-}1^+TIM3^+$ subset. TIM3/PD-1 blockade increased the frequency of $PD1^-$ T-cell subsets bearing characteristics of effector and memory precursor-like cells, indicating that the treatment led to indirect changes in pre-existing populations in the TME. This memory-precursor-like subset requires the transcription factor Tcf7 and shares features with $CD8^+$ T cells that respond to checkpoint blockade in patients [91].

Intratumoural monocytes and macrophages also undergo striking remodelling following anti-ICP mAbs. While $CXC3CR1^+$ $CD206^+$ macrophages – CD206 is a marker of anti-inflammatory M2 macrophages – are present in progressively growing tumours in mice infused with control mAb, they dramatically reduce in response to anti-PD-1 and/or anti-CTLA-4 mAb therapy [80]. The therapy also leads to an accumulation of myeloid cells expressing high levels of *Nos2*

(iNOS), a marker of IFN-γ activated, pro-inflammatory macrophages. Indeed, IFN-γ production, as a consequence of T-cell reinvigoration following anti-ICP mAbs therapy, positively drives polarization of newly arrived monocytes towards iNOS-positive macrophages with anti-tumour activity [80]. In line with this observation, in patients with advanced melanoma treated with nivolumab (anti-PD-1 mAb) therapy, changes in macrophage-associated genes in tumours are associated with better clinical responses, suggesting that macrophages may play an important role in response to anti-ICP mAbs [77].

Several studies have shown that the tumour stroma can be a major target of anti-ICP therapy. As an example, clinical activity of agonist mAb anti-CD40 (developed to mimic CD40L engagement on T cells and to increase T-cell priming) can be a result of anti-CD40-dependent alteration of tumour stroma [92]. In a mouse model of pancreatic ductal adenocarcinoma, anti-CD40 mAb induces tumour regression by the recruitment and activation of circulating macrophages, which then translocate to tumour tissues and degrade the tumour stroma (displaying a decrease in collagen I content, consistent with degradation of the tumour matrix) [92]. Studies also reveal side effects leading to a cytokine storm and lethality, following systemic injection of CD40 agonist antibodies together with IL-2 in aged mice and young obese mice [93, 94]. In these mice, higher percentages of TNF^{+}-activated macrophages are detected in tissues following therapy as compared to young mice. This suggests a link between the hyper-inflammatory cytokine response to systemic immune stimulation and the increase in visceral fat observed in aged or young obese mice [94].

9.3.3 When Therapeutic mAbs are 'Not-So-Good Guys'

The CD40/CD40L story is an interesting case demonstrating that monoclonal antibodies are more than passive immunotherapy agents, and some of them may have multifaceted – beneficial or detrimental – effects on the tumour microenvironment and on anti-tumour immunity.

In a large clinical trial in metastatic colorectal cancer, the addition of cetuximab (anti-EGFR) to bevacizumab (anti-VEGF) plus chemotherapy resulted in decreased progression-free survival [95]. Pander et al. show that M2 macrophages present abundantly in colon carcinoma are activated by cetuximab-opsonized tumour cells, resulting in anti-inflammatory and tumour-promoting factors production, including IL-10 and VEGF. They suggest that this effect might explain the negative clinical effect of cetuximab in colon cancer [96]. In bevacizumab-resistant patient glioblastomas, the therapeutic mAb directly binds to the macrophage migration inhibitory factor (MIF) from the TME and blocks MIF-induced M1 polarization of macrophages, resulting in more M2 pro-tumoral macrophages [97]. Moreover, as VEGF increases glioma MIF production in a VEGFR2-dependent manner, bevacizumab-induced VEFG depletion down-regulates MIF in TME. Nevertheless, it should be noted that other studies in different microenvironments have reported beneficial effects of MIF down-regulation or deletion, including increased intratumoural effector $CD4^{+}$ and $CD8^{+}$ T cells [98, 99], reduced regulatory T cells [98], reduced MDSCs in the tumour [100] and higher numbers of activated DCs [99].

Moreover, mAbs, as therapeutic agents that actively reshape the microenvironment, could in some conditions induce immunosuppressive molecules. It has been reported that the numbers of $CD4^{+}$, $CD8^{+}$ T cells and $CD68^{+}$ macrophages expressing PD-L1 and VISTA inhibitory immune checkpoints increased in the prostate tumour microenvironment after ipilimumab therapy (anti-CTLA-4 mAb) [101]. This suggests that VISTA might represent a compensatory inhibitory pathway in ipilimumab-treated prostate cancer that is poorly responsive to immune checkpoint monotherapy. The authors also show that ipilimumab leads to an increase in PD-$L1^{+}$ and $VISTA^{+}$ macrophages expressing CD163 and ARG1, suggesting a shift towards an M2-like phenotype and function of these cells [101]. Furthermore, whereas antibody-dependent cellu-

lar cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) are two main mechanisms that critically contribute to the efficacy of anti-tumour therapeutic antibodies, a recent study reports that ADCP results in an immunosuppressive phenotype of tumour-associated macrophages with overexpression of inhibitory molecules PD-L1 and indoleamine 2,3-dioxygenase (IDO) [102]. Macrophages that undergo ADCP upon rituximab (anti-CD20) or trastuzumab (anti-Her2/Neu) mAbs treatment subsequently inhibit NK-cell-mediated ADCC and T-cell-mediated cytotoxicity in lymphomas and breast cancers. This study reveals a deleterious role of ADCP in macrophages that can be overcome with concomitant immune checkpoint blockade [102].

Interestingly, different studies also report that FcγR engagement by anti-ICP mAbs dampens anti-tumour activity of the mAbs. In a preclinical model, negative effects of FcγR recruitment was observed for two anti-PD-1 mAbs recognizing different epitopes on PD-1. The mechanism by which FcγR engagement reduces anti-tumour activity is different for the two mAbs. For one mAb, engagement of high affinity activating FcγRI results in the elimination of intratumoural $CD8^+$ effector cells. For the other mAb, the reduced activity relies on its binding to inhibitory FcγRIIB [103]. Anti-PD-1 mAbs (nivolumab, pembrolizumab and cemiplimab) are of the IgG4 isotype which has reduced ADCC and 'null' CDC. However, IgG4 binds to FcγRI and FcγRIIB, and these interactions can have clinical consequences. In vivo imaging studies reveal that a rat IgG2a anti-PD-1 mAb (that is used to mimic the biological property of human IgG4) can be captured from $PD\text{-}1^+$ T-cell surfaces by $PD\text{-}1^-$ tumour-associated macrophages. This transfer limits anti-tumour efficacy of the therapeutic mAb [104]. More recently, hyperprogression observed in cancer patients treated with anti-PD-1 mAbs has been linked to the interactions of the mAbs with $Fc\gamma R^+$ M2 macrophages [105]. A possible role of inhibitory FcγRIIB is suggested by the authors of this work.

These different observations outline deleterious effects of mAbs on anti-tumour immunity, and should be carefully considered for the design of therapeutic strategies in cancer patients.

9.4 Concluding Remarks

Besides the direct impact on tumour growth, mAbs therapies can have remarkable effects on the network of cells within the TME, including (i) induction of long-term anti-tumour adaptive immunity by APC-mediated uptake and presentation of tumour antigens released upon cell death, (ii) durable modulation of the range of immune cells reactive against the tumour and (iii) overall reshaping of the myeloid and lymphoid compartments within the TME (Fig. 9.1a). Bystander effects of therapeutic mAbs can also occur, leading to deleterious inflammation and/or decreased anti-tumour immune responses (Fig. 9.1b). In this case, the underlying mechanisms should be carefully considered to overcome these negative effects with concomitant treatment to reduce inflammatory symptoms or by blocking additional inhibitory pathways.

Immunotherapies in patients with solid tumours include mAbs targeting tumour cells, the tumour vasculature and/or immune cells within the TME. Multiple immune evasion mechanisms can be used by tumours; immunosuppression or exhaustion in the TME, biological or physical barriers around the tumour that inhibit or prevent immune cell infiltration and poor antigen presentation due to a lack of antigens or of antigen-presenting cells. Thus, combinations of antibodies against different targets within the TME can circumvent the current limitations of single antibody therapies. Numerous mAb combinations are under investigation in clinical trials (i.e. antibodies against either different epitopes of the same molecule or different targets on the same tumour cell; anti-angiogenic antibodies combined with tumour-targeting or immunomodulatory mAbs; combinations of antibodies targeting different ICP molecules; anti-ICP mAbs combined with mAbs directed against cytokines, etc.) [106]. Bispecific or multispecific antibodies that simultaneously target tumour cells and immune effector cells are also being currently developed

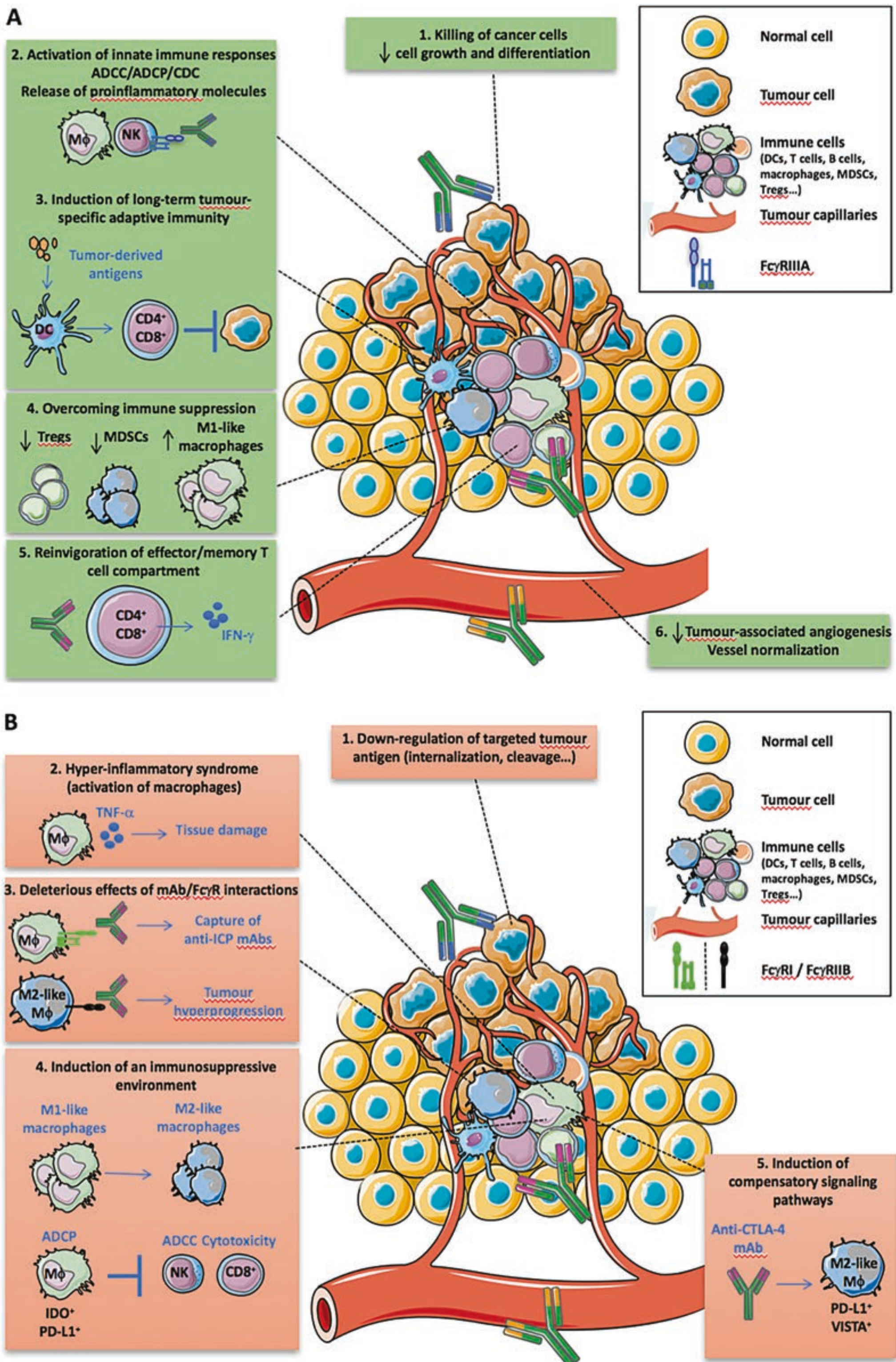

Fig. 9.1 Multifaceted effects of monoclonal antibodies on the tumour microenvironment and on anti-tumour immunity. Different categories of monoclonal antibodies (mAbs) have been developed for cancer therapy. A first category is directed against tumour cells themselves (in blue), a second one comprises antibodies blocking the formation of neo-vasculature that accompanies tumour development (in yellow) and a third category of immunomodulatory antibodies target immune cells in the tumour microenvironment rather than cancer cells (in pink).

for clinical use in patients with solid tumours [106]. These different combinations would exert wider therapeutic effects than a single therapeutic agent. Finally, the categorization of tumours according to the molecular and cellular composition of the TME would help to identify which tumour types are most likely to respond to different types of immunotherapies and to choose the appropriate combination of immunotherapies for each cancer.

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Fig. 9.1 (continued) These antibodies may have beneficial (**a**) or detrimental (**b**) effects on the tumour microenvironment and on anti-tumour immunity. (**a**) 1: Anti-tumour mAbs can block cancer cell activation and/or proliferation or directly induce apoptosis of tumour cells. 2: Besides these direct effects, mAbs recruit C1q molecule (belonging to the complement system) and innate cells expressing receptors for the Fc region of IgG (FcγR), such as NK cells and macrophages (Mϕ), leading to cell lysis and to the formation of tumour cell debris through complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), respectively. 3: Immature DCs then capture the resulting tumour-derived antigens, leading to the priming of self-reactive tumour-specific $CD4^+$ and $CD8^+$ T cells that can act back against tumour cells and eventually circumvent the pro-tumour immunosuppression. 4: MAbs can also have remarkable effects on the network of cells within the TME, including modulation of the frequencies of immunosuppressive cells – such as regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs) – or of anti-tumoural M1-like macrophages. 5: MAbs directed against immune checkpoint (e.g. anti-PD-1, or anti-CTLA-4 mAbs) can restore anti-tumour activity in dysfunctional infiltrating immune cells and induce a shift towards a more activated T-cell compartment that expresses high levels of IFN-γ. 6: Antibodies targeting the VEGF pathway induce a reduction and a normalization of tumour-associated blood vessels (**b**) Different bystander effects of therapeutic mAbs can also occur, leading to deleterious inflammation and/or decreased anti-tumour immune responses. 1: MAbs binding to tumour cells, could be followed by the down-regulation of targeted antigen. Interactions between mAbs and intratumoural macrophages can lead to deleterious effects including 2: hyper-inflammatory syndrome associated with tissue damages (e.g. for anti-CD40 mAbs); 3: decreased therapeutic activity of antibodies directed against immune checkpoint (anti-ICP mAbs) by FcγR-dependent capture of mAbs; or tumour hyperprogression following interactions between pro-tumoral M2-like macrophages expressing FcγR and anti-ICP mAbs. 4: By promoting the differentiation of intratumoural macrophages into pro-tumoural M2-like macrophages or by activating them, anti-EGFR or anti-VEGF mAbs can induce an immunosuppressive microenvironment. Furthermore, macrophages that undergo ADCP upon anti-CD20 and anti-Her2/Neu mAbs treatment can subsequently inhibit NK cell-mediated ADCC and T-cell-mediated cytotoxicity. These macrophages exhibit an immunosuppressive phenotype with overexpression of inhibitory molecules PD-L1 and indoleamine 2,3-dioxygenase (IDO). 5: MAbs blocking immunoregulatory molecules can induce inhibitory compensatory signalling pathways, as reported for anti-CTLA-4 mAb inducing an increased frequency of M2-like macrophages expressing PD-L1 and VISTA inhibitory ICP. (This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com)

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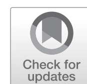

10 Tumor Microenvironment and Nitric Oxide: Concepts and Mechanisms

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Abstract

The cancer tissue exists not as a single entity, but as a combination of different cellular phenotypes which, taken together, dramatically contribute to the entirety of their ecosystem, collectively termed as the tumor microenvironment (TME). The TME is composed of both immune and nonimmune cell types, stromal components, and vasculature—all of which cooperate to promote cancer progression. Not all immune cells, however, are immune-suppressive; some of them can promote the immune microenvironment to fight the invading and uncontrollably dividing cell populations at the initial stages of tumor growth. Yet, many of these processes and cellular phenotypes fall short, and the immune ecosystem more often than not ends up stabilizing in favor of the "resistant" resident cells that begin clonal expansion and may progress to metastatic forms. Stromal components, making up the extracellular matrix and basement membrane, are also not the most innocuous: CAFs embedded throughout secrete proteases that allow the onset of one of the most invasive processes—angiogenesis—through destruction of the ECM and the basement membrane. Vasculature formation, because of angiogenesis, is the largest invader of the TME and the reason metastasis happens. Vasculature is so sporadic and omnipresent in the TME that most drug therapies are mainly focused on stopping this uncontrollable process. As the tumor continues to grow, different processes are constantly supplying it with the ingredients favorable for tumor progression and eventual metastasis. For example, angiogenesis promotes blood vessel formation that will allow the bona fide escape

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of tumor cells to take place. Another process like hypoxia will present itself in several forms throughout the tumor (mild or acute, cycling or permanent), starting mechanisms such as epithelial to mesenchymal transitions (EMT) of resident cells and inadvertently placing the cells in such a stressful condition that production of ROS and DNA damage is unavoidable. DNA damage can induce mutagenicity while allowing resistant cells to survive. This is where drugs and treatments can subsequently suffer in effectiveness. Finally, another molecule has just surfaced as being a very important player in the TME: nitric oxide. Often overlooked and equated with ROS and initially assigned in the category of pathogenic molecules, nitric oxide can definitely do some damage by causing metabolic reprogramming and promotion of immunosuppressive phenotypes at low concentrations. However, its actions seem to be extremely dose-dependent, and this issue has become a hot target of current treatment goals. Shockingly, nitric oxide, although omnipresent in the TME, can have a positive effect on targeting the TME broadly. Thus, while the TME is a myriad of cellular phenotypes and a combination of different tumor-promoting processes, each process is interconnected into one whole: the tumor microenvironment.

Keywords

Tumor microenvironment (TME) · Cancer · Immune surveillance · Angiogenesis · Angiogenic switch · Sprouting angiogenesis · Cancer metabolism · Hypoxia · Nitric oxide · Cancer-associated fibroblasts (CAFs) · Tumor-associated macrophages (TAMs) · Innate and adaptive immunity · Stromal cells · Immunosuppression · Immune elimination/equilibrium/escape · Immunotherapy · Treatment resistance

10.1 Tumor Microenvironment

Long gone is the idea that a tumor is simply a combination of cancer cells that are involved in uncontrolled clonal expansion; instead, there has been a shift to a more revolutionary idea that a tumor is a combination of heterogeneous populations of cells: tumor cells, immune cells and nonimmune cells, stromal components, and vasculature. Together, these create an ecosystem—a cancerous organ-like structure that exists and grows on its own [1–3]. For this reason, the development of current drug therapies has evolved from inhibiting one or many of the specific components that reside in the tumor microenvironment (TME) to the more concrete approach of targeting the tumors broadly [4]. Tumorigenesis is initiated when oncogenic activation disrupts normal gene expression patterns, thereby interrupting normal tissue homeostasis and initiating a secretion of cytokines and growth factors that recruit stromal cells and vascular components [5, 6]. These cells include cancer-associated fibroblasts (CAFs), endothelial cells (ECs), adipocytes, pericytes, and immune cells such as macrophages, monocytes, lymphocytes, and dendritic cells (DCs) that become trapped in the extracellular matrix and are affected by its changing biophysical parameters [7–10]. Thus, the TME is not a static process of resident cell populations but a dynamic and ever-evolving ecosystem that is crucial for the initiation, progression, and metastasis of cancer. To reach significant growth and expansion and establish metastatic niches, the tumor microenvironment involves several important processes that contribute to tumor progression: angiogenesis, hypoxia, endothelial to mesenchymal transition (EMT), macrophage infiltration, and regulatory effects of secreted factors such as reactive oxygen species (ROS) or nitric oxide (NO) (Fig. 10.1) [11–13].

10.1.1 Composition of the Tumor Microenvironment

Cells in the TME are heterogeneous in origin and nature and can come from the bone marrow, blood vessels, or the stroma [14]. The cellular plasticity seen in these cells is mediated by EMT, loss of E-cadherin function, and loss of apical-basal polarity [15]. These cells provide the foundation for the TME.

Stromal Components The stroma is a network of the extracellular matrix (ECM) supported by the basement membrane, which is lined with endothelial cells [16]. The ECM scaffolding is composed of collagen, fibronectin, proteoglycans, and laminins, all of which are intricately interwoven and well organized. The interesting thing about the ECM in tumor tissues is that it has an extremely abnormal morphology—it often exhibits aberrant patterns of fibril deposition, which lead to invasion of the surrounding tissue. Furthermore, the stroma plays a critical role in angiogenesis as it is intertwined with a busy network of blood vessels. As far as cell types residing in the stroma are concerned, these include cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), and tumor-associated macrophages (TAMs). CAFs are known to enhance angiogenesis, tumorigenesis, and metastasis, as well as promote drug resistance. Angiogenesis is usually triggered by CAFs' ability to secrete matrix metalloproteinases (MMPs) and other enzymes that destroy the ECM as well as factors that upregulate expression of the vascular endothelial growth factor (VEGF), which stimulates angiogenesis [17]. On the other hand, MSCs residing in the TME attempt to repair the injured cells by transferring mitochondria via nanotubules but can also differentiate into CAFs, which further promote angiogenesis and metastasis [18]. Thus, while MSCs mean well, in the context of the TME, these cells may actually promote cancer survival and progression. Finally, macrophages are recruited to the TME via signaling molecules and cytokines to fight the rapidly growing ecosystem; however, they can become polarized and converted to TAMs, the M2 phenotype, which actually plays a significant role in cancer progression [19]. Thus, the stroma of the TME is a supportive network that plays an important role in establishing tumor integrity, all the while promoting its subsequent growth and expansion.

Immune Surveillance The main role of the mammalian immune system is to find, tag, and eliminate a pathological invader in order to protect the organism against infectious agents and eliminate damaged cells [20]. However, unlike in normal tissue, cancerous tissue is marked by persistent immunological cell populations that not only expand but also diversify due to malignant processes such as fibrosis, angiogenesis, and neoplasia [21, 22]. Three stages of immune involvement in cancer have been proposed: elimination, equilibrium, and escape [23]. In the first stage, the immune system tags uncontrollably growing cell populations and is particularly efficient at destroying and eliminating them. However, in the equilibrium stage the immune system is not as efficient at fighting the ever-growing malignant cells, giving them sufficient time to adapt to the new immune microenvironment and differentiate into other cell types by undergoing EMT. This allows the establishment of a cancer niche that is full of immune-resistant cells, which will inadvertently develop into a solid tumor. Finally, the involvement of the immune system has been well documented at the escape stage, where it reduces anticancer proteins and other surveillance mechanisms, allowing tumor cells to escape their original niche, migrating to distant metastatic sites. In this sense, the immune system evolves from a mechanism that fights cancer invasion to a mechanism that becomes completely entrapped by the tumor ecosystem and thus promotes cancer progression.

The tumor ecosystem contains cells of both adaptive and innate immunity, both of which play a role in tumor establishment and progression, modulation of angiogenesis, and subsequent immune escape. Adaptive immune cells include T lymphocytes and B cells, while innate immune cells include dendritic cells, natural killer cells, monocytes and macrophages, neutrophils, mast

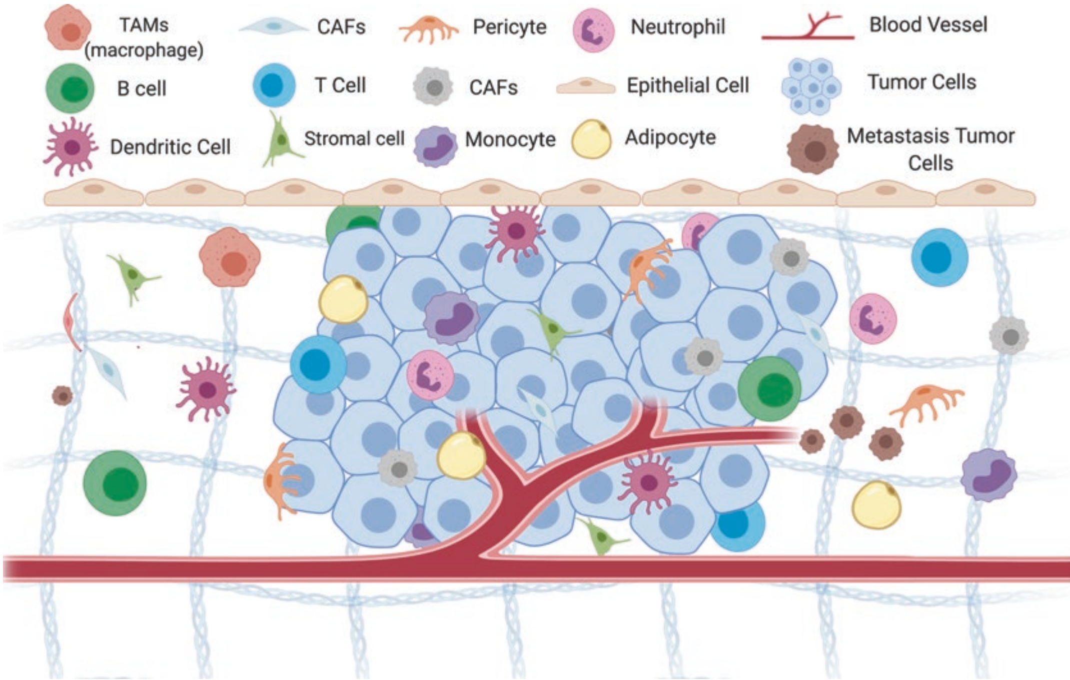

Fig. 10.1 Composition of the tumor microenvironment. TME is a combination of different cellular phenotypes, all of which dramatically contribute to the entirety of their ecosystem. The TME is composed of both immune (T and B cells, dendritic cells, monocytes, TAMs) and nonimmune cells types (CAFs, epithelial cells, etc.), stromal components, and vasculature—all of which exist in unison to allow cancer progression to take place. Not all immune cells, however, are immunosuppressive; some of them can promote the immune microenvironment to fight the invading and uncontrollably dividing cell populations at initial stages of tumor growth. However, many of these processes and cellular phenotypes fall short, and the immune ecosystem more often than not ends up stabilizing in favor of the "resistant" resident cells that begin clonal expansion and may progress to metastatic forms. Stromal components, constituting the extracellular matrix and basement membrane, contain potentially hazardous CAFs, which secrete proteases that initiate angiogenesis through destruction of the extracellular matrix (ECM) and the basement membrane. Vasculature is the biggest invader of the tumor microenvironment, and the reason metastasis occurs. The fusion of the immune and nonimmune cells, stromal components, and vasculature creates a favorable microenvironment for the progression of cancer. TAMs: tumor-associated macrophages; CAFs: cancer-associated fibroblasts

cells, and eosinophils [24]. T cells in the TME can be CD4+ (helper T cells) or CD8+ (cytotoxic T cells), which secrete IFN-gamma, TNF-alpha, and IL-17 that mediate adaptive immune responses and exhibit antitumor effects. T-cell infiltration has been shown to be associated with a positive outcome in cancer patients; however, tumors have evolved to display dominant inhibitory mechanisms that work against proliferation of T effector cells. Currently, a hot target of immunotherapy approaches are immune checkpoint blockade inhibitors, such as cytotoxic T-lymphocyte antigen-4 (CTLA4), programmed cell-death-1 (PD1) and its ligand, PDL1. B-cells, which can be divided into immature B cells, plasma cells, or memory cells, express different immunoglobulins on their surface for antigen recognition, and these phenotypes can vary depending on the stage of tumor as well as tumor type, such as IgM, IgD, IgA, or IgG [22]. In addition, dendritic cells (DCs) express inflammatory cytokines IL-12, IL-23, and IL-1 that promote IFN-gamma CD4+ T-cell responses [25]. Natural killer (NK) cells express HLA class-I receptors, which can recognize and eliminate malignant cells [26]. There are also immunosuppressive cell

types in the TME, which includes the T-regulatory cell population (Treg), myeloid-derived suppressor cells, and M2 macrophages [27–29].

Other Cells In addition to stromal and immune components, there are multiple other cell types residing in the TME that contribute to tumor growth. For instance, endothelial cells continue to grow and divide uncontrollably during cancer progression, which express the VEGF receptor on the cell surface, which allows them to continually stimulate angiogenesis. Platelets within the TME can be an additional source of VEGF and both pro- and antiangiogenic proteins that are usually carried in alpha-granules of platelets [30]. Pericytes are another important cell type that maintains the integrity of blood vessels, but which begin to loosen their attachment upon activation of angiogenesis via signaling molecules such as PDGF, TGF-beta, angiopoietin, and Notch [31]. Loss of pericyte attachment leads to higher permeability of blood vessels and increased metastatic spread of tumor cells.

Vasculature Just like normal tissue, malignant tissue develops a network of blood and lymphatic vessels to supply the necessary oxygen, remove waste and carbon dioxide, and provide a route for immune surveillance [32]. However, unlike in normal tissues, these vascular networks often contain leaky capillaries. During angiogenesis, the vasculature becomes even more complicated. An ever-hypoxic state of the TME initiates aberrant blood vessel formation that allows tumor cells to escape the low-oxygen setting and disseminate to distant sites, where nutrient and oxygen levels are not yet depleted. Hypoxia triggers the release of hypoxia-inducible factor 1 (HIF-1), leading to upregulation of genes such as VEGF and PDGF, which stimulate angiogenic factors [33]. Formation of new blood vessels begins with degradation of the basement membrane around the tumor and disruption of the EC monolayer, followed by tube formation and EC invasion into the surrounding tissue [34]. Pericyte recruitment then stabilizes the newly formed blood vessels, providing structural support and allowing for the necessary crosstalk between ECs that further stimulates VEGF production [35].

10.2 Angiogenesis

10.2.1 Angiogenic Switch

In the absence of new vasculature, tumor growth is restricted with a well-maintained balance between proliferation and apoptosis [36]. An angiogenic switch occurs when this homeostasis—the balance between proangiogenic and angiogenic pathways—skews in one direction over the other. This loss of angiogenic homeostasis may occur for several reasons, but evidence from many studies points to genetic and epigenetic remodeling as being the main contributors to such a switch. The angiogenic switch is correlated with both loss of tumor suppressor genes, such as p53, and upregulation of oncogenes, such as Myc, which increases production of VEGF by 10-fold [37–39]. Regardless of the reasons this happens, angiogenic switch starts a cascade of processes that make it much more likely and favorable for cancer to progress.

Hypoxia is a well-known inducer of angiogenic switch, as it forces a very rapid metabolic reprogramming, skewing this well-maintained angiogenic homeostasis [40]. In the absence of oxygen, cells go into a crisis mode, trying to get nutrients and oxygen from nearby tissues. Tumors are no exception to this—they are highly hypoxic structures with abnormal vascular networks that are constantly trying to survive. Hypoxia shifts the cellular metabolism in a way that the extracellular space becomes more acidic and glucose metabolism along with lactic acid production become upregulated, which subsequently lowers the pH in the TME [41]. Such a pH decrease is correlated with rigorous EMT, cell dissemination, and eventual metastasis [42–45]. An acidic environment is an important contributor to an increase in angiogenic factors through upregulated expression of VEGF [46]. Hypoxia triggers additional processes, ranging from mobilization of bone-marrow-derived precursor cells to

immune activation [47]. Hypoxia is also one of the main contributors to induce expression of VEGF, MMPs, and angiopoietin-like 4 (ANGPTL4)—all of which are promoters of angiogenic pathways [48, 49].

10.2.2 Mechanisms That Drive Angiogenesis

Pathological vessel proliferation is one hallmark of cancer progression [50]. In normal tissue, blood vessels appear as ordered tubular networks that facilitate the transport of gases, nutrients, and cells around the body, and are carriers of different trophic signals, all of which are necessary for normal organ homeostasis [51]. They are categorized into veins, arteries, and capillaries, and comprise a thin monolayer of epithelial cells on the luminal side, the basement membrane on the outside covered with pericyte, and vascular smooth muscle cells. Two processes are required for the maintenance of vascular networks, namely, vasculogenesis and sprouting angiogenesis, both of which are essential mechanisms for cancer progression. Vasculogenesis is the de novo formation of new blood vessels, while sprouting angiogenesis is the formation of new vessels from a pre-existing network of capillaries.

Sprouting angiogenesis is the first process in blood vessel formation, which involves an intricate interplay between the ECM, stromal cells, and soluble factors [52]. During sprouting angiogenesis, endothelial cells begin to loosen their contact with pericytes, which are the stabilizing cells surrounding blood vessels, whose function it is to maintain the vessels' integrity and quiescent state. Once endothelial cells have been destabilized, they undergo EMT, where they acquire a highly migratory and invasive personality. This process is accompanied by the basement membrane destabilization and ECM degradation, much needed for angiogenesis to proceed by allowing the formation of an immature blood vessel [53]. Vessel maturation occurs when a process known as mesenchymal to endothelial transition (the reverse of EMT) occurs, which restores endothelial cells to their quiescent state, followed by the synthesis of a new basement membrane [54]. Initiation of an angiogenic sprout is controlled by VEGF and the Notch signaling pathway [55]. The growing end of the sprout is known as the "tip cells," which respond to VEGF signaling by extending filopodia that sense their environment and recruit stromal cells for stabilization and support. Endothelial cells that are located in the stalk portion of the angiogenic sprout are known as the "stalk cells," which undergo the same process but sprout sideways, contributing to extensive branching—most often in response to VEGF-A signaling [56].

Vasculogenesis begins with the mobilization of endothelial progenitor cells (EPCs), which get recruited in response to chemokines, cytokines, and growth factors released by both tumor and stromal cells [57]. In hypoxic conditions, expression of HIF is seen to activate VEGF, PDGF, C-X-C chemokine receptor Type 4 (CXCR4), and stromal-derived factor-1 (SDF-1), which are important for EPC proliferation [57, 58]. In response to VEGF and PDGF particularly, EPC mobilization occurs through the release of matrix metalloprotease 9 (MMP9), which activates the Kit ligand, a stem-cell migratory cytokine that allows EPC mobilization to take place [59]. Besides its role in primary tumor growth, vasculogenesis has also been implicated in the dissemination of cells and eventual metastasis via soluble factors such as SDF-1, which recruit EPCs to distant sites [60]. The interaction of SDF-1 on EPCs and the CXCR4 receptor on tumor cells establishes the development of a pre-metastatic niche.

10.2.3 Metastasis Due to Angiogenesis

Unfortunately, angiogenesis is the main contributor to cancer progression from a primary tumor ecosystem to a metastatic tumor ecosystem, where cells disseminate and invade the surrounding tissue. As already discussed, VEGF is the main inducer of multiple processes that make metastasis much more likely—it upregulates pro-

tease production that degrades the basement membrane and secretes factors that weaken endothelial-tumor cell interactions, a necessary process for metastasis [61]. The pericyte lining the blood vessels also loosens their attachments to endothelial cells on the luminal side of the vessel, leading to a decrease in endothelial cell survival and creation of a leaky environment through the intercellular gaps that allow tumor cells to escape and travel to disseminated sites [62–64].

10.2.4 Blocking Vessels in the TME

Since angiogenesis is such an important part of neoplasms, modern therapies have focused on finding a suitable therapy that targets this process. There has even been marginal success in the treatment of several tumor types with such drugs as Sutent and Avastin against kidney and colorectal cancer [50, 65–67]. However, modern approaches still rely on standard chemotherapy, which seems to fall short due to its low selectivity of cancer cells and its high toxicity to normal cells [68]. While drug delivery to tumors is inefficient because of highly abnormal vasculature, as already discussed, multiple targets are being developed to inhibit or induce regression of neoplastic blood vessels [69].

Direct vessel signaling inhibition. EPC mobilization and seeding are the absolute requirement necessary to start angiogenesis, which occurs via targeting of tyrosine kinase (TK) receptors by angiogenic growth factors such as VEGF [70, 71]. Therefore, approaches that inhibit TK receptors or their ligands are being investigated as an antiangiogenic therapy approach, including antibodies, soluble factors, and small-molecule inhibitors [71–73]. Examples of TK inhibitors (TKIs) include Sorafinib, which downregulates Raf signaling along with VEGFR-2 and PDGFR-beta [71], and Sunitinib, a TKI for both VEGFR-2 and PDGFR-beta and a potent inhibitor of c-kit [72].

Vascular environment inhibition. Another approach is to inhibit the vascular environment of the TME, and since angiogenesis begins with EPC recruitment and establishment of EPC metastatic niches, this process may also target pharmacologically. For instance, as the SDF-1/CXCR4 signaling axis is the main regulator of EPC mobilization and homing, antibodies against CXCR4 might be a plausible target [60].

Vessel normalization. Another promising type of treatment is actually the opposite of the two aforementioned therapeutic approaches—a desire to stabilize vascular networks [74]. As already mentioned, in contrast to normal vasculature in nonmalignant tissue, which is efficient and follows predictable patterns, the vasculature of a tumor is in a state of extreme disarray, characterized by aberrant, disorganized, and dilated morphologies. This decreases pericyte association, elevates chances for hypoxia, increases permeability to escaping tumor cells, and lowers perfusion. One of the main issues of chemotherapeutic drugs and immune therapies is that they cannot reach the target area because of this faulty vasculature [75]. Thus, drugs have developed to stabilize the vascular networks, with the goal of improving pericyte recruitment and tightening cell-to-cell junctions in a process known as vascular normalization [76]. Such drugs include bevacizumab (Avastin) and trebananib, which have shown favorable clinical outcomes when used in combination with chemotherapy in breast and ovarian cancer patients [77–79].

10.3 Hypoxia

10.3.1 Role of Hypoxia in the TME

Hypoxia is at the forefront of cancer growth and progression [80]. Because of oncogene activation, initial cell proliferation is so aggressive that there are not enough available nutrients and oxygen in the environment to supply the cells, and so the environment becomes hypoxic as those resources quickly deplete [81]. This lack of nutrient and oxygen supply triggers a cascade of changes in the TME that increases production of angiogenic factors and revascularization events [82]. However, as already mentioned, vascular structures in the tumor environment are not perfectly ordered; instead, they are chaotic and

sporadic with constant angiogenic mechanisms triggered in response to hypoxic episodes, which lead to vascular leakiness and nonlaminar blood flow [83, 84]. Because tumors are heterogeneous structures with dynamic fluctuations in blood flow, within a single tumor ecosystem there may exist regions of both mild hypoxia and acute hypoxia; those fluctuations in blood flow can lead to cycling hypoxia, which can vary from hours to days. Two frequencies of cycling may be detected: higher-frequency cycling usually results from small alterations in red blood cell perfusion, while lower-frequency cycling results from large-scale remodeling of the vascular network and angiogenesis [85]. Short-term hypoxia activates autophagy as well as apoptotic and metabolic adaptation of cells to survive in adverse conditions [86, 87] and production of reactive oxygen species (ROS), which contributes to tumor survival and growth [88, 89]. Acute hypoxia induces metastasis and is associated with aggressive tumor phenotypes [90]. Long-term hypoxia contributes to long-term cellular and genetic changes, such as DNA breaks, higher DNA replication errors, genetic instability, and mutagenesis [91–93]. Regardless, neither chronic nor acute hypoxia is good news for a growing tumor—these sporadic events at irregular intervals usually present with adverse clinical manifestations.

10.3.2 Hypoxia in Blood Vessel Formation

Hypoxia induces overexpression of transcription factors such as HIF-1-alpha and HIF-2-alpha (Fig. 10.2), which target blood vessel formation and metastasis, and play a role in resistance to treatment [94]. Abnormal angiogenesis ensues in response to the pathological condition in which, because of rapid cell proliferation, nutrients and oxygen are used up by the rapid cell increase [95, 96]. The hypoxic state allows the production of proangiogenic factors, thus skewing the intricate balance that maintains the normal angiogenic equilibrium, resulting in rapid vessel formation. These disordered vessels lack structure, organization, and proper pericyte contacts, which make them leaky and more susceptible to metastatic spread. Thus, angiogenesis results from a cell's attempt to relieve the hypoxic state, thus inducing the formation of more blood vessels to relieve the oxygen demands, but inadvertently restarting the vicious cycle [51]. However, the cycle continues as soon as another need to improve hypoxia arises. There are some antiangiogenic drug therapies being developed that target highly malignant and invasive cancer types, including bevacizumab, an anti-VEGF monoclonal antibody approved for colorectal cancer and other solid tumor types [97].

10.3.3 Hypoxia in Metastasis

A bona fide metastatic process results from hypoxia-induced angiogenesis, where the cells end up escaping the highly hypoxic conditions via the newly formed blood vessels to relieve oxygen demands and survive [51]. However, as the result of sporadic growth, the new vasculature is so fragile, highly permeable, and heterogeneous that it permits the massive relocation and delivery of tumor cells to distant organs via circulation. Levels of tumor oxygenation and overexpression of HIF-alpha has been shown to correlate with highly metastatic and aggressive tumors and the poor overall survival of patients [98]. It may not come as a surprise, therefore, that previous hypoxic cells can also keep their ability to metastasize at a higher rate than cells only cultured in normoxic conditions, as was shown by an orthotopic mouse model, where lymph node metastasis seemed to increase due to acute hypoxia followed by normoxia [99]. Mechanistically, hypoxia seems to trigger an invasive and migratory phenotype of cells by inducing EMT [100, 101]. On the genetic regulatory level, genes responsible for maintaining an epithelial phenotype are reduced (E-cad, beta-catenin) [102], while mesenchymal-like gene expression is stimulated (N-cad, vimentin, SMA, CXCR4) [103, 104]. Though the bona fide master regulator of the physiological EMT is TGF-beta, it is increased in response to hypoxia, activating

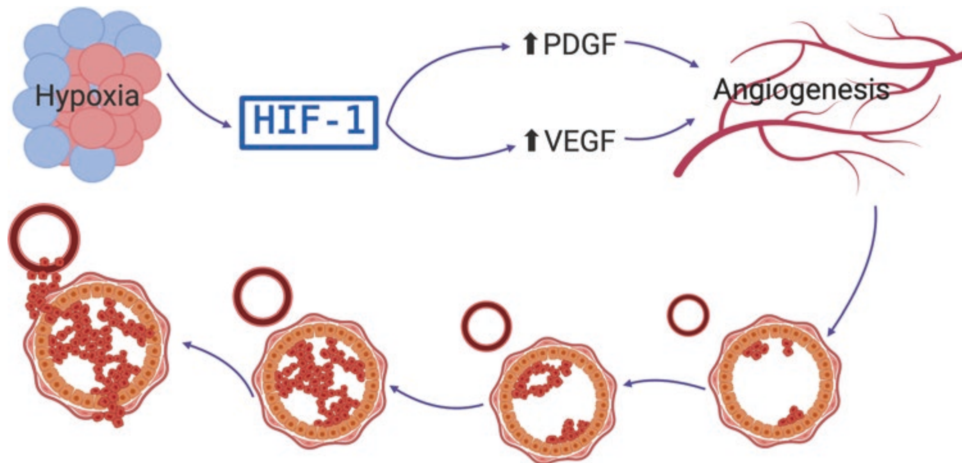

Fig. 10.2 Role of hypoxia in the TME. Hypoxia can present itself in several forms throughout the tumor—mild or acute, cycling or permanent—initiating mechanisms such as epithelial to mesenchymal transitions (EMT) of resident cells as well as inadvertently placing the cells in such a stressful condition that production of ROS and DNA damage is unavoidable. The reason DNA damage to mild forms of hypoxia (or cycling hypoxia) is so dangerous is because it can induce mutagenicity while allowing resistant cells to survive. This is where drugs and treatments can subsequently suffer in effectiveness. Hypoxia causes release of inducible factor 1 (HIF-1) that upregulates vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) that stimulates angiogenesis. Angiogenesis degrades the basement membrane, disturbs the endothelial cell (EC) monolayer, and results in an invasion into the surrounding tissue

downstream transcription factors (TFs) such as Snail, Smad, Slug, and Twist with the inhibition of E-cadherin expression [105], thus inducing massive EMT. The development of resistance to radio- and chemotherapy has been linked to faulty EMT processes that are regulated via Snail and Slug [105]. HIF inhibition has been viewed as a major promising therapeutic approach, especially for metastatic and solid tumor types, and there has been marginal success with several drugs that have undergone phase I and II clinical trials [106, 107]. Thus, there are multiple correlations between metastasis, development of drug resistance, and hypoxia-induced EMT changes that take place in terminal cancers.

10.3.4 Hypoxia in Radiation and Drug Resistance

Resistance to treatment-induced apoptosis from radio- or chemotherapy is one of the biggest obstacles in cancer treatment [108]. Often, this occurs because residual cells that are resistant to treatment are left over and multiply, contributing to a clonal expansion of treatment-resistant cells that can quickly lead to tumor recurrence and metastasis [109]. Hypoxia can also cause resistance of cancer cells to treatment, often leading to various physiological states that allow cells to survive via a variety of mechanisms such as cell cycle arrest (quiescence), a state of reduced proliferation that protects cells from external stress, inhibition of apoptosis, senescence, autophagy, and increased mitochondrial activity [110–112]. In normoxic conditions, an abundance of oxygen supply causes oxygen to react with free radicals generated by ionizing radiation during treatment in a process known as “oxygen fixation,” which leads to irreversible DNA damage and profound cell death [113]. However, when oxygen supplies are low, there is a slow generation of free radicals that would otherwise contribute to DNA damage, allowing cells to adapt and survive. These “leftover” cell populations after treatment are dangerous because they can come back at full force. An additional disadvantage is that radio- or chemotherapy often targets the bulk of rapidly proliferating cells. Hypoxic cells are difficult to target because they are usually quiescent, low-

proliferating, have stem-cell-like properties, and live in the most hypoxic (innermost) regions [105, 112]. The least sensitive cell cycle phases to ionizing radiation are G1 and the end of S phase, while the most sensitive are G2 and M, when DNA repair mechanisms are most susceptible [114]. Since these facts about hypoxia-induced treatment resistance have surfaced, researchers have turned to attempting to block HIF-1 with inhibitors to stimulate the cells to respond to treatment in the same way that normoxic cells do. For instance, the HIF-1 inhibitor (YC-1) was tested in tumor-bearing mice and found to cause radiation-induced vessel damage, while HIF1-alpha inhibitor (PX-478) re-sensitized squamous and pancreatic cancer cells, cultured in a hypoxic environment, to radiation therapy [115, 116].

10.4 Nitric Oxide

10.4.1 Nitric Oxide in the Tumor Microenvironment

Nitric oxide (NO) is an intriguing molecule that has resurfaced in the recent decade after much debate as to its pathogenicity. NO, however, is also a known inducer of apoptosis and may play a therapeutic role in cancer rather than just a pathological one [11]. Thus, NO has a dual role as both a physiological and a pathophysiological molecule. NO is a product of a metabolic reaction that converts L-arginine to L-citrulline using nitric oxide synthase (NOS), and can exist in several forms depending on the origin of its production: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [12]. NO by itself is reactive and thus has been implicated to play a significant role in activating multiple signaling pathways. It is regulated by intracellular calcium concentrations (nNOS and eNOS), but it can also be brought about with no calcium present (iNOS) by the upregulation of factors such as endotoxins, inflammatory cytokines, hypoxia, and oxidative stress [12, 117]. Overexpression of different NOS isoforms has been linked to many solid tumors [13]. The most striking feature of NO is that it can exhibit a dose-dependency, so that at high concentrations it acts as the source of nitrosative and oxidative stress, causing DNA damage and mitochondrial dysfunction along with upregulating apoptosis, while at low concentrations it decreases apoptosis and promotes angiogenesis, thus displaying tumoricidal roles (Fig. 10.3) [11, 118]. However, because of its obvious antitumor effects, NO has been gaining popularity in anticancer treatments. For instance, as already discussed, resistance to chemo- and radiotherapy is a main issue in metastatic forms of cancer, but NO has been shown to sensitize cells to subsequent treatment, thus providing a combinatorial therapy approach to cancer treatment [119].

Besides promoting many of the TME essential processes (angiogenesis, metabolism, apoptosis), NO might also play an important role in reprogramming the immune component of the TME.

10.4.2 NO in Immunosuppression

NO can play the role of an immunosuppressive messenger in the TME. One of the main immune cell populations that NO targets is T-cell-mediated antihumoral responses by mediating several mechanisms. In one study, it was shown that NO-derived peroxinitrite inhibits T-cell proliferation, a mechanism which consequently induces apoptosis of T cells [120]. NO can also interfere with T-cell humoral recognition by inhibiting migration of T cells into the TME. One explanation for this interesting observation could be that high concentrations of NO in the TME induces S-nitrosylation of CCL2, a chemoattractant chemokine, which abolishes the tumor's ability to attract CD8+ T cells into the tumor core [121]. In addition, there is another population of cells regulated and attracted by CCL2—myeloid-derived suppressor cells (MDSCs), which produce NO and thus further restrict T-cell migration into the tumor by downregulating E-selectin [122]. INOS was also shown to promote recruitment of T-regulatory cells (Tregs), an immunosuppressive cell type, by modulating IL-12 expression [123]. Additional studies have pointed

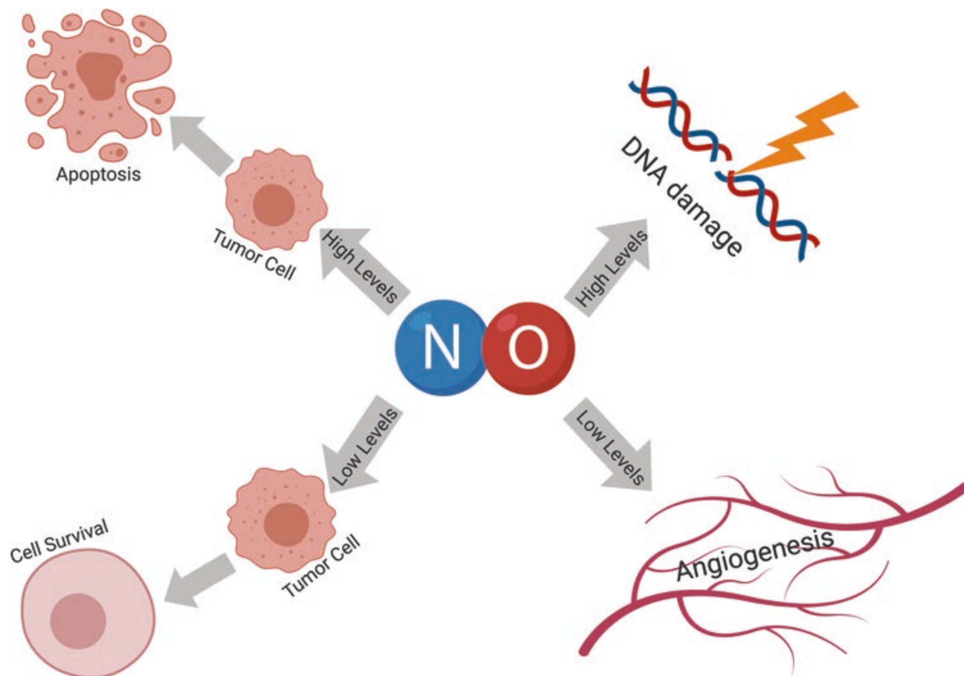

Fig. 10.3 Role of nitric oxide in the TME. Nitric oxide (NO), previously grouped with ROS in the pathogenic molecule category, was recently found to be an important molecule in the TME. Though NO has been found to induce damage by causing metabolic reprogramming and the promotion of immunosuppressive phenotypes, it has also been found to have a positive effect on targeting the TME broadly. Furthermore, it has been found to be exceptionally dose-dependent with regard to both its negative and positive effects on the TME. NO at low doses decreases apoptosis along with promoting angiogenesis, while at high doses it causes DNA damage and increases apoptosis

to other mechanisms of NO-mediated tumor immunosuppression, such as inhibiting antigen presentation from dendritic cells to CD4+ helper T cells [124] and directly impairing natural killer (NK) cell functions [125]. It may also play a role in immune-activation processes, as NO was shown to be released by activated macrophages in the TME, thus inducing their cytotoxic antitumor activity [126].

10.4.3 NO in Evasion of the Immune Response by Cancer Stem Cells

Considering all of this, there is a large body of evidence that points to NO also exerting other immunosuppressive functions on the TME by regulating the "stemness" of cancer cells. Tumors seem to be so good at evading immune system recognition because of a subset of cells in the TME termed "cancer stem cells," and this has led scientists to refer to cancer as a "stem cell disease" [127]. The "cancer stem cell model" states that there is a subpopulation of cancer stem cells (CSCs) at the initiation stages of tumor growth, which display pluripotent and renewing properties. These properties allow the initial tumor seeding events to take place and eventual propagation and metastasis, which are responsible for the bulk of failures of many conventional therapies and poor cancer survival rates [128, 129]. The effect of stem cell signaling on the TME seems to be driven by the active WNT/beta-catenin signaling pathway and a complete absence of T-cell gene expression signature in human melanoma [130]. In addition, CSCs do not exhibit tumor antigen expression and show a

defective MHC-antigen presentation pathway and downregulation of MHC class I molecules [131]. CSCs can also recruit cells that further promote immunosuppressive functions, supporting the CSC phenotype and stabilizing their niche in the TME [132].

NO metabolism contributes to the maintenance of "stemness" that is characteristic of CSCs. As was shown in glioblastoma, eNOS activates the Notch signaling pathway, which promotes the CSC phenotype [133]. CSCs also promote expression of the iNOS isoform, which cranks up the synthesis of NO [134]. The maintenance of the CSC phenotype by NO signaling is demonstrated in several cancer types, including breast [135], colorectal [136], lung [137], and liver [138] cancers. NO produced by immunosuppressor cells in the TME may contribute to the plasticity of cancer cells themselves that allows them to gain and maintain a stem cell phenotype [139].

10.4.4 Metabolic Reprogramming by NO in the TME

Tumors adapt rapidly to stress conditions by rewiring their metabolic pathways. Shockingly, most energy in the TME is generally derived from aerobic glycolysis, which is not as efficient at producing ATP but is a fast process that can generate some energy to be used immediately. Unfortunately, the downside is that aerobic glycolysis quickly builds up lactic acid in the extracellular space, lowering the pH [140]. The acidic microenvironment induces expression of VEGF that, besides increasing angiogenesis, also leads to polarization of the M2 macrophage phenotype [141]. At early stages of tumor growth, TAMs maintain a proinflammatory and antitumorigenic phenotype, the M1 state, while at later stages of metastasis and tumor progression M1 differentiates into the M2 phenotype, which displays a protumoral phenotype and contributes to immunosuppression [142]. In high-grade tumors, TAMs are mostly the M2 phenotype, which also produces NO and has endogenous mechanisms that protect tumor cells from chemotherapy [143]. Since hypoxia induces the upregulation of enzymes involved in glycolysis and the inhibition of mitochondrial function, in this sense, NO-induced hypoxia contributes to the "Warburg effect" (aerobic glycolysis metabolism observed in cancer) [144]. NO has also been shown to prevent differentiation of M1 macrophages into the M2 phenotype by abolishing mitochondrial respiration and reducing their plasticity [145].

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